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Title The Purification and Characterization of a Soluble Mitochondrial Adenosinetriphosphatase from Cabbage, Brassica oleracea

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A soluble enzyme from cabbage mitochondria possessing adenosinetriphosphatase (ATPase) activity has been purified 15-fold over the activity present in whole mitochondria. The enzyme has been characterized with respect to stability limits, substrate specificity, the effect of various activators and inhibitors, metal ion requirement, incubation temperature, enzyme concentration, time of incubation, and substrate concentration. The lack of the capacity to catalyze the adenosinetriphosphate-inorganic phosphate ^{32} (ATP-Pi^{32}) exchange reaction was demonstrated.

Two procedures were developed for the enzyme preparation, both of which effected a 15-fold purification. The first method involved a one-step ammonium sulfate precipitation of the soluble fraction obtained after 105,000 x g centrifugation of sonic-comminuted cabbage mitochondria. A second procedure employed the initial preparation of a mitochondrial acetone powder followed by n-butanol and aqueous solvent extraction with subsequent fractionation by diethylaminoethyl (DEAE)-cellulose column chromatography.

The cabbage mitochondria were isolated by differential centrifugation of tissue homogenates and a method was developed for the isolation of large quantities of mitochondria required for preparation of cabbage mitochondrial acetone powder. The latter was found to be a convenient source of the enzyme and could be stored at -18°C. for at least a month without significant loss of enzyme activity.

Evidence is presented which suggests the possible occurrence of an intermediate in oxidative phosphorylation in DEAE-cellulose eluants of aqueous acetone powder extracts from cabbage mitochondria only. The enzyme solutions possessing ATPase activity were shown to contain a non-dialyzable, acid labile source of inorganic phosphate.

The purified enzyme prepared by either method was found to exhibit at least two, and possibly three pH optima, suggesting the presence of several different ATPase activities in the intact mitochondrion. The enzyme demonstrated a lack of substrate specificity, hydrolyzing the nucleoside-5'-triphosphates of guanine, cytosine, inosine and uridine, as well as ATP. The enzyme also exhibited inorganic pyrophosphatase activity. Significant levels of apyrase or other enzyme activities catalyzing the production of AMP from ATP were shown to be absent in the purified enzyme, although detectable levels of AMP were present in reaction mixtures with less purified enzyme preparations.

Adenosinediphosphate (ADP) was shown to inhibit the enzyme, an observation consistent with the finding that the ATPase activity was markedly stimulated in the presence of an ATP regenerating system.

The enzyme was dependent upon the presence of Mg^{++} for maximal activity and was found to be stimulated by 2,4-dichlorophenoxyacetic acid (2,4-D) in the range 1×10^{-6} M to 1×10^{-5} M; by 2,4-dinitrophenol (DNP) between 1×10^{-5} M and 5×10^{-5} M; and by 1×10^{-3} M oleic acid, whereas the ATPase of intact cabbage mitochondria, although

stimulated by oleic acid, was not stimulated by 2,4-D or DNP.

The purified ATPase was shown to lose activity rapidly at physiological pH but could be markedly stabilized at either lower (pH 6.0) or higher (pH 8.5) pH values. The presence of a sulfhydryl reagent (cysteine or mercaptoethanol) and ATP was also found to increase the enzyme's stability during storage at 4°C. The ATPase activity was completely destroyed by freezing, at pH values less than 5.2 or greater than 9.5, or by heating to temperatures in excess of 45°C. The presence of protective agents such as ATP, dimethylsulfoxide (DMS), glycerol, glutathione, cysteine, mercaptoethanol, 2,3-dimercaptopropanol (BAL), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid did not protect the enzyme from the freezing treatment, although ATP was of limited value in preserving enzyme activity up to 55°C. ATP in combination with cysteine protected the enzyme activity to pH values as low as 4.7.

The purified enzyme could be kept as a precipitate in 70% ammonium sulfate at pH 6.0 and at 4° C. in the presence of 1×10^{-3} M ATP and 5×10^{-3} M cysteine or mercaptoethanol for at least a week without significant decline in activity.

It was necessary to remove these protective agents by Sephadex column chromatography before incubating the enzyme in order to elicit maximum activity. Dialysis of the enzyme resulted in appreciable loss of activity.

Some of the results presented here are at variance with the results obtained from studies on the ATPase activity of intact cabbage mitochondria and suggest that the structural association of the protein with the intact mitochondrion may impart different characteristics to the enzyme than those present in the soluble phase freed from the complexities of the mitochondrial milieu.

THE PURIFICATION AND CHARACTERIZATION OF A SOLUBLE MITOCHONDRIAL ADENOSINETRIPHOSPHATASE FROM CABBAGE, BRASSICA OLERACEA

by

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Dedicated to the
Memory of

MARY E. PETERSEN
(1904-1962)

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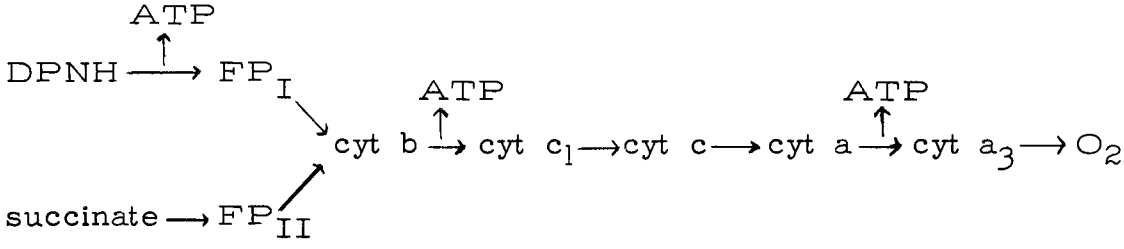
INTRODUCTION

The living cell derives a major portion of the energy required to drive its metabolic processes from the hydrolysis of adenosinetriphosphate (ATP) to adenosinediphosphate (ADP) and inorganic phosphate. The rather specific role played by ATP as the sole product of oxidative, photosynthetic and glycolytic phosphorylation has elicited in recent years the interest of a wide variety of researchers in a massive effort to understand more completely the enzymatic machinery and mechanistic pathways associated with this important molecule.

The mitochondrion is known to be a major locus for the production of ATP in the cell since the presence of enzymatic activities catalyzing the reactions of the Krebs cycle (67, p. 893-903), fatty acid oxidation (31, p. 957-972) and oxidative phosphorylation (61, p. 565-570) have been unequivocally confirmed. Although the existence of mitochondria in animal tissues has been known for more than 60 years (19, p. 13-42) and their presence in plant cells since the work of Meves (52, p. 264-268) in 1904, it has only recently been possible to isolate these particles with any reasonable degree of facility (66, p. 169-178; 6, p. 619-635).

In recent years the study of mitochondria by

investigators from many fields has accelerated to staggering proportions. The current trend of research appears to be leaning in the direction of detailed investigations of submitochondrial fragments, with the ultimate goal of elucidating the structure and function of mitochondria at the molecular level. Thus, mitochondria have been comminuted by treatment with digitonin (14, p. 489-506; 18, p. 507-518), ethanol (76, p. 80-85; 24, p. 516-524), or sonic oscillation (51, p. 219-229) to produce particles capable of catalyzing oxidative phosphorylation. These results and others, particularly those obtained from the kinetic studies of Chance and collaborators (9, p. 383-393; 10, p. 395-407) have led to a formulation for the respiratory chain in animal mitochondria which may be represented as (8, p. 123-129):



The probable sites of coupled phosphorylations on the chain are as shown (41, p. 450-456).

Cooper and Lehninger have proposed a mechanism for

the coupling of phosphorylation with electron transport as follows[1] (16, p. 561-578):

(1) $AH_2 + C \longrightarrow AH_2\text{-}C$

(2) $AH_2\text{-}C + B \longrightarrow A \sim C + BH_2$

(3) $A \sim C + Pi \longrightarrow A + P \sim C$

(4) $P \sim C + ADP \longrightarrow ATP + C$

Other workers have proposed similar schemes.

Meyers and Slater (54, p. 558-572) proposed a scheme essentially the same as the one given above except that the high energy intermediate $A \sim C$ is written in the reduced instead of the oxidized form. Likewise, Chance et al. (11, p. 439-451), in agreement with Meyers and Slater, have suggested a mechanism involving a reduced compound, $AH_2 \sim C$, instead of the oxidized form.

The mechanisms depicted above represent the three main schools of thought concerning the nature of oxidative phosphorylation. Proposals such as the above have been worked out largely from the study of mammalian systems. Much less is known about oxidative phosphorylation in plant

[1] AH_2 and BH_2, A and B, are respiratory chain carriers, in the reduced and oxidized forms, respectively. C is an enzyme, and $\sim$ represents the "high energy bond".

mitochondria. The electron transport system of plant mitochondria appears to be similar to that of various animal systems. Spectrophotometric evidence indicates that flavoprotein and cytochromes of the b, c, and a type participate in the oxidation of reduced diphosphopyridine nucleotide (DPNH) and succinate. In most cases the system is susceptible to the same inhibitors as animal systems and, indeed, oxidative phosphorylation in many plant tissues including mung bean (22, p. 247-256), castor bean (1, p. 115-118), spinach (40, p. 199-214), lupine (13, p. 23-32), and cabbage (21, p. 374-376) has been shown to reach the levels found in animal systems. Thus, there can be little doubt that the energy-trapping mechanisms found in plants can be as efficient as those found in animals.

It is a generally accepted theory that the mitochondrial adenosinetriphosphatase (ATPase) is functionally integrated into the enzymatic mechanism of oxidative phosphorylation. Two distinct types of ATPases are believed to exist: Mg^{++}-stimulated ATPase and 2,4-dinitrophenol (DNP)-stimulated ATPase. In the above mechanism, the DNP-stimulated ATPase is believed to involve the breakdown of A C to A and C. (74, p. 1589-1597). This can be interpreted as a reversal of

Reaction (4) followed by reversal of Reaction (3) and the decomposition of A∞C. The adenosinetriphosphate- inorganic phosphate^{32} (ATP-P^{32}) exchange reaction can be represented by Reactions (3) and (4) while the adenosinetriphosphate-adenosinediphosphate (ATP-ADP) exchange is defined only by Reaction (4).

Available evidence so far reported is consistent with the view that the DNP-stimulated ATPase, the ATP-P^{32} exchange and the ATP-ADP exchange reactions represent part of the process of oxidative phosphorylation. The role of the Mg^{++}-stimulated ATPase in oxidative phosphorylation is not known. Cooper (17, p. 484-491) has evidence which suggests that DNP-stimulated ATPase may in fact be due to an effect of DNP on the Mg^{++}-stimulated ATPase, and that in reality the two ATPases may be considered as one. Siekevitz *et al.* (69, p. 378-391) likewise conclude that the Mg^{++}-activated ATPase constitutes a part of the enzyme sequence involved in the DNP-activated ATPase.

ATPase activity has been studied in numerous plant and animal preparations but only relatively few studies have been made with soluble systems. Meyerhof and Ohlmeyer (49, p. 11-17) purified a soluble Mg^{++}-stimulated ATPase from

yeast 50-fold. The enzyme was found to be specific for the terminal phosphate group of ATP. Barbato and Alpert (4, p. 255-261) have recently achieved a 200-fold purified, cholesterol-free, Mg^{++}-dependent soluble ATPase from rat muscle. The enzyme is believed to be the basic lipoprotein moiety of the particulate ATPase purified earlier by Kielley and Meyerhof (32, p. 588-591) but freed from cholesterol.

A 100-fold purified soluble, DNP-stimulated ATPase from beef heart mitochondria which requires Mg^{++} for maximum activity has been shown to be an obligatory participant in oxidative phosphorylation by Pullman et al. (64, p. 3322-3329). The data are consistent with the view that the Mg^{++}-stimulated ATPase, as well as the Mg^{++} plus DNP-activated enzyme, is the same protein.

Although the evidence favoring only one ATPase activity is no doubt significant, the evidence in the literature favoring the existence of two distinct enzymes cannot be disregarded. It is, for example, difficult to reconcile the differences in nucleotide specificity and pH optima, as well as the preferential inactivation of the DNP-stimulated activity with the existence of only a single protein (15, p. 547-560; 16, p. 561-578). Thus, it becomes necessary to modify the reaction sequence

for the hypothesis described above in order to explain the role of a Mg^{++}-stimulated ATPase in oxidative phosphorylation. The modified scheme can be depicted as

$$(3)\quad A\sim C + Pi \longleftrightarrow A + P\sim C$$

$$(3')\quad P\sim C + Y \longleftrightarrow C + P\sim Y$$

$$(4')\quad P\sim Y + ADP \longleftrightarrow Y + ATP$$

where Y indicates another enzyme.

In the above scheme the reversal of Reactions (4') and (3'), followed by hydrolysis of $P\sim C$, would explain the Mg^{++}-stimulated ATPase, and in a similar fashion the DNP-stimulated ATPase would be given by the reversal of Reactions (4'), (3') and (3), followed by the breakdown of $A\sim C$.

Mammalian mitochondrial systems are sensitive to the presence of DNP. Thus, it has been shown (7, p. 369-377) that low concentrations of this substance in mitochondrial suspensions completely uncouples phosphorylation from respiration, stimulates ATPase, inhibits the ATP-P^{32} and ATP-ADP exchange reactions and releases respiration in the absence of a phosphate acceptor.

The effect of DNP on plant mitochondria has not been studied as extensively as with mammalian systems, although

there are numerous reports which show that DNP uncouples oxidative phosphorylation in plant mitochondrial preparations (25, p. 27-32; 6, p. 135-148; 1, p. 115-118).

There is much less known about the effects of DNP on plant mitochondrial ATPase. DNP has been found to be without effect on mitochondria isolated from cauliflower (40, p. 199-214), but Forti (20, p. 898-909) has demonstrated DNP stimulation of ATPase in pea mitochondria. The stimulation required the obligatory participation of low concentrations of phosphate (1×10^{-3} M). This has been the only demonstration of a definite stimulation by DNP of plant mitochondrial ATPase.

There has been considerable interest in mitochondrial ATPase in recent years. Lardy and Elvehjem (38, p. 1-30) were the first to suggest that the ATPase of mitochondria might represent a partial reversal of oxidative phosphorylation and since then, numerous studies have been made on mitochondrial ATPase in efforts to explain the role of the enzyme in the phosphorylation process. It is a well-known observation that intact mitochondria show practically no ATPase activity, but that the "latency" of the enzyme is released following disruption of the mitochondrial integrity by, for example, aging (37, p. 485-500), DNP (29, p. 297-330),

or by sonic treatment (34, p. 448-449). Kielley and Kielley (36, p. 213-222) suggested that since fresh mitochondria contain ADP, it might account for the "latency" or "masking" of the ATPase. Since these authors also observed a marked inhibition of ATPase activity with ADP, this reasoning may be of some significance in view of the recent experiments of Gatt and Racker (23, p. 1015-1023) in which the continuous removal of ADP with phosphoenol pyruvate and pyruvate kinase resulted in a 4 to 5-fold stimulation of the ATPase activity in freshly isolated liver mitochondria.

There have been only a few reports of detailed studies of mitochondrial ATPase in the literature. A Mg^{++}-activated, particulate ATPase activity has been purified from disintegrated mitochondria by Kielley and Kielley (36, p. 213-222). The enzyme was specific for the terminal phosphate group of ATP. Lardy and Wellman (39, p. 357-370) purified a soluble enzyme activity from aqueous extracts of acetone-dessicated liver mitochondria which required Mg^{++} and would hydrolyze ATP. Addition of DNP greatly enhanced the ATPase activity. Forti (20, p. 898-909) has extensively studied the ATPase activity of pea mitochondria. Only the terminal phosphate was split by the enzyme and the cleavage

was stimulated by DNP in the presence of 1×10^{-3} M phosphate.

The most recent detailed study of mitochondrial ATPase has been presented by Pullman et al. (64, p. 3322-3329), in which a 100-fold purification of the enzyme from beef heart mitochondria is described. The soluble enzyme is irreversibly cold labile at 4° C. but remains active at room temperature. The enzyme cleaves only the terminal phosphate group of ATP and is inhibited by ADP. This is the only example of a highly purified and completely characterized soluble ATPase that has been reported, although in a preliminary report Selwyn (68, p. 4) has succeeded in purifying a soluble ATPase from aqueous extracts of acetone dried rat and beef liver mitochondria which has properties similar to those described for the soluble ATPase of Pullman et al. (64, p. 3322-3329), including the curious cold lability property.

There have been no reports in the literature of any attempt to purify and characterize a soluble ATPase activity from plant mitochondria. Results from this laboratory have shown that intact cabbage mitochondria demonstrate a high respiratory activity (21, p. 374-376) and recently a significant soluble ATPase activity has been obtained from comminuting

cabbage mitochondria.

It thus was of interest to attempt a further purification of this activity and to study the properties of this system isolated from the complexities of a particulate preparation. The enzyme was isolated from cabbage mitochondria prepared by a modification of the method described by Freebairn and Remmert (21, p. 374-376). Purification of the ATPase activity from cabbage mitochondria was achieved either by DEAE-cellulose chromotography of aqueous acetone powder extracts or by ammonium sulfate fractionation of soluble extracts obtained from sonically disrupted mitochondria. The enzyme has been characterized with respect to stability, optimal conditions for maximum activity, substrate specificity, metal ion requirement, lack of ATP-P^{32} exchange activity, stoichiometery of reaction, pH optima and the efforts of added DNP, oleic acid and 2,4-D. Some of the results reported suggest that the soluble enzyme activity exhibits markedly different properties from the ATPase associated with the intact mitochondrion, and that these aberrations may be due, in part, to a fundamental difference in structural environment of the enzyme elicited during the course of the purification procedure. Evidence is also presented which suggests that

the mitochondrial membrane may be an important factor in determining the nature of the ATPase activity associated with these organelles.

METHODS AND MATERIALS

Isolation of Cabbage Mitochondria

The mitochondria were isolated from cabbage, Brassica oleracea, var. Capitata L., by modifications of the procedure described by Freebairn and Remmert (21, p. 374-376). Two procedures were used for the isolation of the mitochondria. The first of these was suitable for small-scale preparations, and the second method was employed for large-scale preparations.

Small-Scale Preparation. Fresh cabbage heads were purchased wholesale from a local market and stored in a cold room maintained at 4° C. The cabbages were used within two days following their purchase. Inner portions of the heads were quartered and the inner core of each segment was removed. The remaining parts were then chopped into a finely divided mass which was weighed into 250-gram portions. Each portion was blended with 60 ml. of homogenizing medium[2] in a Waring blender. The mixture was homogenized

[2] The homogenizing medium contained 1.0 M sucrose, 0.1 M potassium phosphate, and 0.01 M ethylenediaminetetraacetate (EDTA), pH 7.4.

during 5 short bursts (1-1-1-5-2 seconds) of the blender motor, each burst being accompanied by a vigorous rocking of the blender bowl assembly in order to facilitate contact of the blender blades with the cabbage tissue. After the blending operation, each homogenate was strained through 8 layers of #40 cheesecloth into a previously chilled beaker. All homogenizing operations were carried out in a cold room maintained at 4° C. The pH of the homogenate was 6.9-7.1. The homogenate was centrifuged at 3,000 x g for 10 minutes to remove intact cells, chloroplasts and other debris. The supernatant fraction was retained and centrifuged at 12,000 x g for 10 minutes to sediment the mitochondria. The mitochondrial pellet was washed once with 400 ml. of 0.25 M sucrose for each 250 g. of cabbage originally used by suspending and recentrifuging at 12,000 x g. All centrifugations were carried out in a Servall refrigerated centrifuge. The washed mitochondrial pellet was finally suspended in glass redistilled water with a Potter-Elvehjem tissue homogenizer.

Large-scale Preparation. The above method was found to be impractical for the large-scale preparation of mitochondrial acetone powder and an alternative procedure was developed.

In this second method the individual cabbage heads were quartered as before, but the inner core was not removed. A typical preparation involved 160 lbs. of cabbage which was chopped with 6.0 l. of homogenizing medium into a thick slurry in a Fitzmill Model D Comminuting machine. The homogenate was collected in a 50 l. aluminum vessel immersed in an ice bath at 0° C. The homogenizing operation itself was carried out at room temperature, but the temperature of the homogenate could be kept lower than 10° C. if the homogenizing medium, cabbage, and collection vessel were previously cooled at 4°C. before comminution. The mash was immediately transported to a cold room at 4°C. and the liquid portion recovered by squeezing suitable portions through two layers of heavy cottom fabric into large glass vessels, the last amount of liquid being removed by use of a hydraulic press.

From 50-55 l. of extract were routinely obtained by this procedure. The liquid was poured through 4 layers of #40 cheesecloth to remove any large debris present and then centrifuged in an International 4-liter refrigerated centrifuge at 2,000 rpm for 10 minutes to sediment cells, chloroplasts and debris. The supernatant of this fraction was then

centrifuged at 13,200 x g for 30 minutes to sediment the mitochondria. The mitochondria were washed once with a total volume of 600 ml. of 0.05 M tris-maleate buffer, pH 7.4, and recentrifuged at 13,200 x g for 30 minutes. The centrifugation employed for the sedimentation of the mitochondria was carried out in a Servall refrigerated centrifuge kept between 0° C. and 5° C. throughout the operation. The washed mitochondrial pellet was finally suspended in 2.0 l. of 0.05 M tris-maleate buffer, pH 6.0, with a 100 ml. Potter-Elvehjem homogenizer fitted with a teflon plunger.

In developing a suitable procedure for isolating large quantities of cabbage mitochondria, it was found that the most suitable, although highly laborious, method was to employ several Servall centrifuges at once. Attempts at centrifuging the extract in a Sharples 18-liter centrifugal centrifuge or by employing a Szent-Györgyi-Blum continuous flow centrifuge attachment were unsuccessful. This failure may have been due in part to the somewhat viscous and foamy nature of the extract.

Sonic Treatment of the Mitochondria

A 10-KC Raytheon sonic oscillator model DF 101 was

used in all sonication studies. The mitochondria were sonicated at 4° C. in a volume of 100-110 ml. glass redistilled water and then centrifuged either for 10 minutes at 20,000 x g followed by centrifugation at 105,000 x g for 60 minutes or by eliminating the 20,000 x g spin and centrifuging only at 105,000 x g. The slower centrifugation speed was employed when it was desired to study the disrupted mitochondrial membrane fragments, whereas only the 105,000 x g spin was used when the soluble fraction was under consideration.

During the latter stages of this study it was found that the enzyme exhibited maximum stability in the presence of a sulfhydryl reagent and at pH 6.0. The enzyme, therefore, was prepared by the sonic disruption of mitochondria at pH 6.0 and in the presence of 5×10^{-3} M cysteine or mercaptoethanol.

Acetone Powder Preparation from the Mitochondria

The mitochondrial suspension contained in 2 l. of 0.05 M tris-maleate buffer was adjusted to pH 6.0-6.2 with 1.0 M acetic acid and then added slowly to 10 volumes of acetone held at -18°C. The acetone was stirred vigorously with a magnetic stirrer during the entire operation. The

addition of the mitochondrial suspension was carried out over a period of 30 minutes, after which stirring was continued for an additional 30 minutes. The light-colored precipitate was allowed to settle, and the clear dark-green supernatant was decanted off and discarded. The residue was transferred to a Büchner funnel and washed twice with 200 ml. acetone at -18° C. and twice with the same volume of ether at -18° C. The tan clay-like product was then transferred to several layers of paper towels and allowed to dry at room temperature for 10 minutes. Last traces of moisture and organic solvents were removed by transferring the residue to a dessicator containing P_2O_5 and paraffin chips and storing in vacuo at -18° C. for 8 hours. The material was ground to a fine powder in a mortar and pestle at room temperature and stored in a tightly stoppered jar at -18° C. until used. Yields of 60-65 g. of mitochondrial acetone powder could be prepared routinely in this manner.

Purification of the ATPase Activity

Two methods were developed whereby a 15-fold increase in ATPase activity over that present in the intact cabbage mitochondria could be effected. The procedures

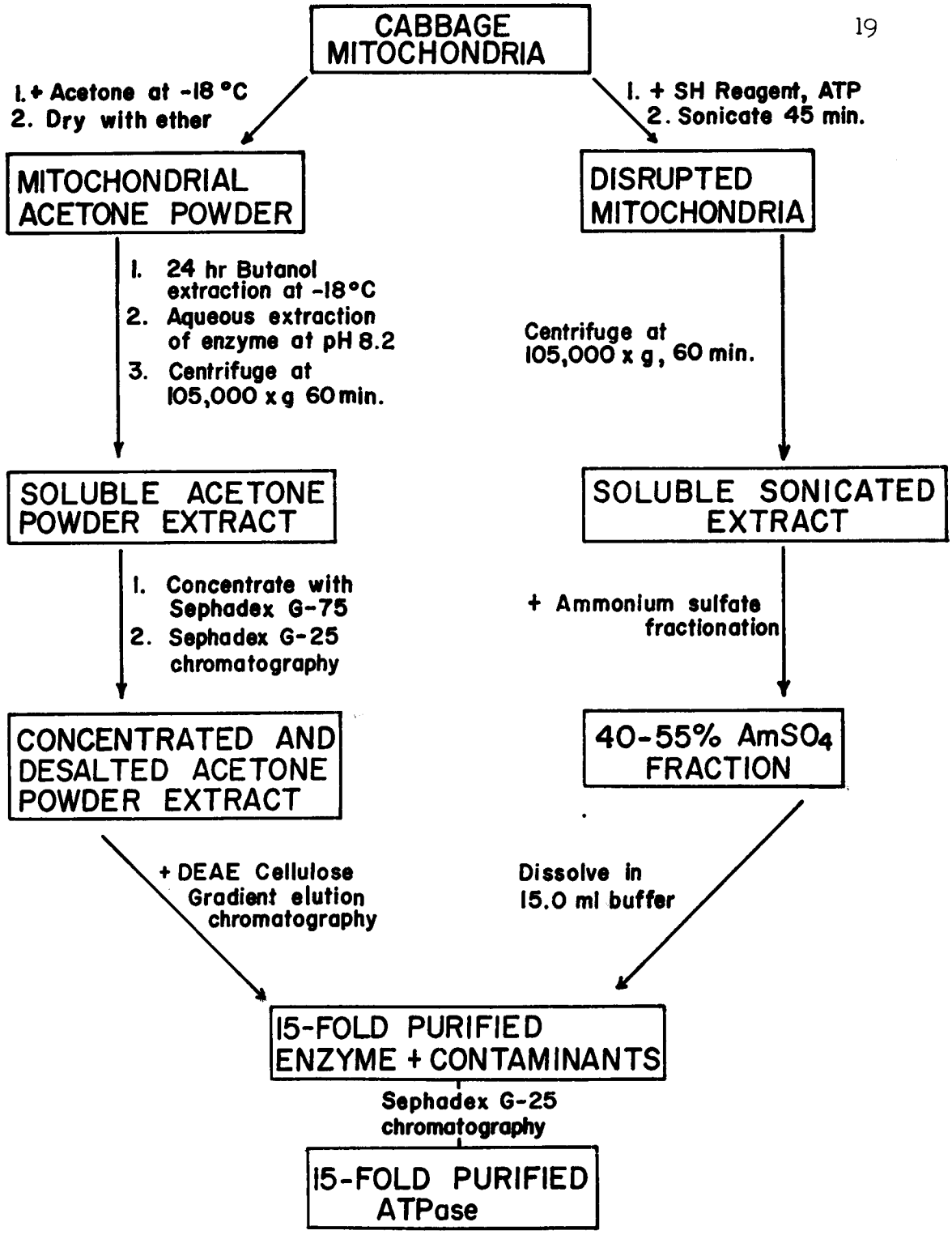


FIGURE 1

Summary of Procedures Used During ATPase Purification

employed are summarized in Figure 1. One method employed ammonium sulfate fractionation of the 105,000 x g supernatant fraction from sonically disrupted mitochondria, while an alternative procedure involved DEAE-cellulose chromatography of soluble extracts from the mitochondrial acetone powder.

Purification from Sonically Disrupted Mitochondria. Mitochondria were isolated as described earlier. The pellet from 2 kg. cabbage was suspended in 100 ml. of a solution containing 0.01 M tris-acetate buffer, pH 7.2, 5×10^{-3} M cysteine and 1×10^{-3} M ATP. The pH was adjusted to 6.0-6.2 with 1 M acetic acid, and the suspension was subjected to sonic treatment for 45 minutes at 0° C. The liquid was centrifuged 30 minutes at 105,000 x g, after which the clear supernatant was decanted and the pellet reextracted once with 15 ml. of the original suspension medium. The pH was adjusted to 6.2 and the suspension centrifuged as before. The supernatant fractions from both centrifugations were combined, giving a final volume of 100-105 ml. containing 3.0-3.5 mg. protein/ml. Centrifugation was carried out in a Spinco Model L preparative centrifuge maintained between 0° C. and 5° C. 100 Ml. of the cooled extract was placed in a 400 ml. beaker and dry reagent grade ammonium sulfate was added slowly, with

stirring, until 40% saturation was reached. The solution was allowed to equilibrate with gentle stirring for 20 minutes and finally centrifuged at 12,000 x g for 10 minutes. The residue was discarded and ammonium sulfate was added to the supernatant fraction as before until 55% saturation was reached. After equilibration and centrifugation as before, the residue was taken up in 15 ml. of 0.05 M tris-acetate buffer, pH 7.2. Usually a small turbidity resulted and was removed by centrifugation at 12,000 x g for 5 minutes. The supernatant fraction from the second ammonium sulfate precipitation was discarded. The solution of the 40-55% ammonium sulfate fraction was placed on a 1 x 30 cm. column of Sepadex G-25, coarse grade, previously equilibrated with 300 ml. of 0.05 M tris-acetate buffer, pH 7.2. The enzyme was eluted with the same buffer. The flow rate was 65-70 ml./hr. The operation was facilitated by the fact that the enzyme migrated as a sharp yellow band down the column. As the band just reached the lower orifice of the column, fractions were collected in suitable containers. Usually the first 15 or 20 ml. contained most of the enzyme activity and was free of interferring ions. The enzyme prepared in this manner was approximately 15-fold purified. All steps during the purification were carried

out at 0° C. to 5° C.

Purification from Acetone Powder. The dried acetone powder was first extracted 24 hours with n-butanol at -18° C. as follows: For each gram of acetone powder in a mortar, 3 ml. of n-butanol were added and the mixture ground with a pestle until a homogeneous consistency was attained. Following the initial grinding, the mixture was agitated with an overhead stirrer for the balance of the extraction period. The entire operation was performed at -18° C. and all apparatus used was pre-chilled to that temperature before use. The mixture was then poured directly into a Büchner funnel previously chilled to -18° C. and washed with 10 volumes of acetone at the same temperature. This was followed by washing with three volumes of dry ethyl ether at -18° C. and then drying at room temperature for 10 minutes. The n-butanol-extracted acetone powder was transferred to a dessicator and dried at -18° C. over P_2O_5 and paraffin chips in vacuo for 8 hours, as described earlier. For routine enzyme preparations, 6.0 grams of the n-butanol-extracted acetone powder were mixed in a 100 ml. mortar with 15-20 g. of "Ottawa" sand previously washed in one volume 1×10^{-3} M EDTA followed by 10 washings with glass redistilled water.

The dry mixture was vigorously ground with a pestle for several minutes until a finely divided, homogenous mixture was obtained. This grinding step and all others were carried out in an ice bath maintained at 0° C. Small portions (10-15 ml.) of extraction medium[3] were added, followed by gentle grinding. After a total of 30 ml. of extraction medium were added, the mixture was centrifuged at 12,000 x g for 10 minutes and the supernatant liquid decanted into a chilled 100 ml. graduated cylinder. The residue was extracted again with 30 ml. extraction medium except that the process was carried out in a centrifuge tube and the mixing of the residue with the extraction medium was accomplished with the aid of a thick glass stirring rod. Following centrifugation and decantation as before, the process was repeated a third time with sufficient extraction medium to give a final volume of extract totaling 80 ml. The pooled supernatant fraction was centrifuged at 105,000 x g for 60 minutes and the clear, yellow supernatant liquid retained. The pH of this fraction was 8.0-8.2. The extract was transferred directly into a 5/8" diameter visking dialysis bag and immersed in sufficient dry Sephadex

[3] The extraction medium contained: 0.05 M $KHCO_3$; 0.05 M tris-acetate buffer, pH 8.2; 5 x 10^{-3} cysteine; and 1 x 10^{-3} M ATP. The pH was 8.2.

G-75 to cover the bag completely. This procedure effected a reasonably rapid and efficient concentration of the protein solution. The concentration was allowed to proceed for 6 hours at 4° C. This treatment invariably resulted in a 30-35% reduction in volume with no loss in protein. Usually 50-55 ml. of extract containing 7-8 mg. protein/ml. was recovered with no loss in enzyme activity. The enzyme solution was then exchanged on a 2.2 x 60 cm. column of Sephadex G-25 (coarse grade) previously equilibrated with 0.01 M tris-HCl buffer, pH 8.2. 25-30 Ml. of the extract was placed on the column and eluted with 0.01 M tris-HCl buffer in a similar fashion described for the first purification procedure. The first 25 ml. of eluate containing 4-5 mg. protein/ml. were collected following the appearance of the yellow enzyme band at the lower end of the column. This solution was immediately transferred to a 2.2 x 60 cm. column of DEAE-cellulose (medium mesh) previously equilibrated with 0.01 M tris-HCl buffer, pH 8.2. During this operation the remaining 25-30 ml. of enzyme extract was exchanged for 0.01 M tris-HCl, pH 8.2, on the regenerated column of Sephadex G-25 in the same manner as before. The recovered enzyme solution was similarly placed on the same DEAE-cellulose

column after the first aliquot was adsorbed. The adsorption process was facilitated by the application of 5-10 lbs/in^2 nitrogen gas. Linear gradient elution of the adsorbed enzyme was begun as soon as the latter was totally adsorbed on the column. Traces of the enzyme solution adhering to the walls of the column were washed into the cellulose with a few ml. of buffer. The elution technique employed was that described by McGilvery (50, p. 141-150) and adapted to a NaCl-tris-HCl-mercaptoethanol elution system. The concentration gradient was from 0 to 0.6 M NaCl in 0.01 M tris-Hcl, pH 8.2. Mercaptoethanol was present at a concentration of 5×10^{-3} M. The flow rate was adjusted to 60 ml./hr. with nitrogen gas pressure, corresponding to a pressure of about 25 cm. Hg above atmospheric pressure. The eluate was collected in 5 ml. fractions in a LKB 3400 B RadiRac fraction collector. The enzyme usually appeared in fractions 60-70 and a preliminary ATPase assay was required to establish the location of the most active fractions. This was facilitated by the fact that, as was the case in the first purification procedure, the enzyme activity was closely associated with an intense yellow color. All chromatographic operations were carried out in a cold room maintained at 0°-4° C.

Preparation of DEAE-Cellulose Columns

DEAE-cellulose was prepared for packing into the columns by the method described by Peterson and Sober (59, p. 3-27). The DEAE-cellulose was suspended in sufficient 0.01 M tris-HCl buffer, pH 8.2, to make a thick slurry which was poured slowly into a 2 x 60 cm. glass column fitted with a frittered glass disc and with a Teflon stopcock assembly. Packing of the cellulose was facilitated with a pressure of 10 lbs/in^2 nitrogen gas. Following the packing, a circular disc of Teflon sponge was placed on top of the cellulose surface to eliminate disturbances of the topmost layer of adsorbant during the application of the protein solution. After packing, the column was washed with at least 2 liters of 0.01 M tris-HCl buffer, pH 8.2, before the addition of the enzyme preparation. The packing step was carried out at room temperature and, when completed, the packed column was transferred into the 4° C. cold room and allowed to reach temperature equilibrium for at least 4 hours before use. It was found necessary to repack the column after each fractionation, since considerable portions of adsorbed protein could not be removed even with very concentrated salt solutions and the use of basic solutions resulted in

pronounced swelling of the cellulose bed. Regeneration of the DEAE-cellulose was carried out as described earlier, and the columns were repacked in the same manner as before.

Preparation of Sephadex Columns

The Sephadex was prepared for packing into columns according to the method described by Porath and Flodin (62, p. 1657-1679). The columns employed were of the same design as those described above for the DEAE-cellulose chromatography. Coarse grade Sephadex was used in all column work as it afforded much more rapid flow rates without significant loss in resolving power. The columns were operated at atmospheric pressure with a hydrostatic head sufficient to give a flow rate of 65-70 ml./hour. After packing the columns at room temperature as before, the latter were allowed to reach temperature equilibrium in a cold room at 4° C. for 4 hours before use. The columns were washed with a least two liters of buffer before attempting desalting operations or buffer exchange. Repacking of the Sephadex columns was not necessary as the residual protein could be removed by flushing with sufficient quantities of buffer before using again.

Characterization and Assay of the ATPase Activity

Enzyme solutions eluted from the DEAE-cellulose column were assayed for activity by incubating for 10 minutes with a solution containing 10 μmoles/ml. ATP, 5 μmoles/ml. $MgCl_2$ and 100 μmoles/ml. tris-HCl buffer, pH 7.2. 1.0 Ml. aliquots of the enzyme solutions were pipeted into flasks containing 1.0 ml. of the above reagents which previously had equilibrated for 5 minutes at 30° C. After incubation the enzymic reaction was stopped by pipetting 1.0 ml. of cold 0.8 M trichloroacetic acid (TCA) into the reaction vessels. The solutions were immediately filtered to remove precipitated protein and then assayed for inorganic phosphate. Initial inorganic phosphate concentration was determined by removing aliquots from certain reaction vessels at zero time. Inorganic phosphate was determined by a modification of the method of Martin and Doty (47, p. 965-967) and as described by Lotlikar (45, p. 18-20).

The ammonium molybdate reagent was prepared as described by Martin and Doty (47, p. 965-967); the standard phosphorus solution was prepared as described by Pons, Stansbury, and Hoffpauir (60, p. 492-503). The acidified ethanol reagent was prepared according to the method

described by Lotikar (45, p. 19). The Fiske-Subbarow reagent was prepared according to Umbreit et al. (73, p. 273).

Unless otherwise indicated, all characterization studies on the purified enzyme were carried out with an incubation mixture consisting of 20 μmoles ATP, 10 μmoles $MgCl_2$ and 200 μmoles tris-acetate buffer, pH 7.2, and 1 ml. of enzyme containing 0.9-1.0 mg. protein/ml. in a total volume of 2.0 ml. Protein was determined by the Biuret method as modified by Jacobs, et al. (30, p. 147-156). The ATPase activity was estimated as the amount of inorganic phosphate formed during incubation at 30° C. for 20 minutes. The enzymic reaction was stopped by the addition of 1.0 ml. cold 0.8 M TCA to the reaction vessels. Inorganic phosphate was determined in the same manner as describer earlier.

The ATP-regenerating system used in the enzyme characterization was that described by Gatt and Racker (23, p. 1015-1023). Pyruvic kinase was diluted 1:10 with 0.01 M tris-acetate buffer, pH 7.2, and dialyzed 6 hours at 4° C. against 2 liters of the same buffer at pH 7.2 before addition to the reaction vessels.

For the ATP-P^{32} exchange study, the incubation

medium was the same as that described for measuring ATPase activity, except that an amount of P^{32} equivalent to about 1×10^6 counts/min./flask was also present. All other conditions were the same as described earlier for the ATPase study.

P^{32} incorporation into ATP was determined by the method of Nielson and Lehninger (56, p. 555-570).

For the chromatography of adenine nucleotides the procedure described by Wadkins and Lehninger (74, p. 1589-1597) was used.

Materials

The ATP-disodium salt (from muscle), UTP, GTP and CTP tetrasodium salts (source not specified), ITP trisodium salt (from muscle), ADP-sodium salt (from muscle), AMP (from muscle), phosphoenol pyruvate, cysteine, BAL, pyruvic kinase (crude) and DEAE cellulose were obtained from the Sigma Chemical Company. 2,4-Dinitrophenol, isobutanol, mercaptoethanol and 2,4-D were obtained from Eastman Kodak Company. EDTA (reagent grade), acetone (c.p. grade), ether (c.p. grade), and n-butanol (reagent grade) were obtained from Baker Chemical Company. Oleic acid (U.S.P. grade) was the product of Matheson, Coleman and Bell. P^{32} was obtained as $H_3P^{32}O_4$ from the Oak Ridge National Laboratory. Sephadex G-25 and G-75 were obtained from Pharmicia, Upsala Sweden. All other chemicals used were of reagent grade.

RESULTS AND DISCUSSION

Part I

Solubilization of ATPase Activity from Cabbage Mitochondria by Sonic Treatment

High frequency sonic oscillations were first shown by Hogeboom and Schneider (28, p. 302-303) to be a useful means of disrupting liver mitochondria. More recently, Beyer (5, p. 552-553) has reported the successful release of ATPase activity into the soluble fraction by sonic treatment of rat-liver mitochondria. He observed that two different ATPase activities were solubilized: a Mg^{++}-stimulated enzyme and a Mg^{++}-independent enzyme. Application of high frequency sonic vibrations to suspensions of cabbage mitochondria resulted in a similar release of ATPase activity into the soluble fraction.

Optimum Sonic Treatment Time. A first approach to the purification of the ATPase activity from sonic-comminuted cabbage mitochondria was to determine the optimum sonic treatment time for the recovery of the solubilized enzyme. Previous experiments (58) had demonstrated that the best mitochondrial suspension medium was distilled water. It is

possible that the hypotonic medium facilitated the fragmentation of the mitochondrial membranes during the sonic treatment. The disposition of the ATPase activity from various fractions of sonically treated cabbage mitochondria is shown in Table I. It is clear that the amount of solubilized ATPase activity steadily rises with the duration of sonic treatment and levels off near 40 minutes. The particulate activity can be seen to rise steadily, paralleling a similar increase in the soluble fraction. This suggests that the particulate activity is being "solubilized" and moreover, that the solubilization process may involve a preliminary exposure of catalytic sites on the particulate protein during the course of the sonic disruption process.

Conditions for Maximum ATPase Stability. Later studies on the characterization of the enzyme showed that the activity could be stabilized considerably in the presence of 5×10^{-3} M cysteine or mercaptoethanol and 1×10^{-3} M ATP and at pH values near 6.0. Sonic treatment of mitochondria was therefore carried out with these modifications during subsequent enzyme preparations, since under these conditions there was generally a significant increase in the soluble ATPase activity following sonic treatment. It is possible that the

TABLE I

The Effect of Sonication Time on the Solubilization of ATPase Activity from Intact Cabbage Mitochondria

Sonication Time Minutes	ATPase Activity (MμMoles Pi formed/mg. Protein/30 min.)	
	Particulate Fraction	Soluble Fraction
0	10.5	0.99
5	12.3	1.59
15	20.4	3.80
25	18.5	3.82
40	8.6	8.31
60	7.3	6.70

Each flask contained: 30 μmoles ATP, pH 7.4; 300 μmoles tris-maleate buffer, pH 7.4; 30 μmoles $MgCl_2$; 15 mg. serum albumin; 1.0 ml. mitochondria enzyme preparation containing 14.9 mg. Protein/ml; and distilled water to a final volume of 3.0 ml.

Incubation was for 30 minutes at 30° C.

presence of the sulfhydryl reagent, ATP, and acid conditions served to reduce denaturation effects inherent in the sonic communition process.

Part II

Preparation and Extraction of a Soluble ATPase Activity from Mitochondrial Acetone Powder

In 1953 Lardy and Wellman (39, p. 357-370) succeeded in extracting a soluble ATPase activity from rat-liver mitochondria which had been dessicated with acetone. More recently, Selwyn (68, p. 4) has obtained highly purified soluble ATPase activities from aqueous extracts of beef heart and rat liver acetone powders. In a similar fashion, the successful preparation of a stable acetone powder from cabbage mitochondria containing a readily extractable ATPase activity was achieved in this laboratory.

Mitochondrial Preparation. Early attempts at producing a suitable acetone powder from mitochondrial suspensions in 0.25 M sucrose were unsuccessful, as the precipitated protein became enmeshed in a gummy residue which could not be further dessicated. Substituting distilled water or dilute buffer

for 0.25 M sucrose in the wash step for the preparation of mitochondria described earlier would have doubtlessly resulted in significant mitochondrial swelling and possible rupture, the result being a "leaching out" of soluble factors into the aqueous phase. To eliminate this possibility, the wash step was dispensed with; the mitochondrial pellet was worked up with dilute buffer (0.05 M tris-acetate, pH 7.2) instead of sucrose and added directly to the acetone following adjustment of the pH to 6.0-6.2 with 1.0 M acetic acid. Any enzyme "leakage" would thus be retained in the suspending medium and could be effectively recovered during the dessication process. This procedure was found to be satisfactory for preparing a stable, dry powder as described earlier.

The pH of the mitochondrial suspension during the dessication process was found to have a significant effect on the activity of the enzyme as can be seen in Table II.

Removal of Acetone. It was found that in the final stages of the acetone powder preparation, washing with dry ethyl ether greatly facilitated the removal of acetone. No inactivation of the enzyme occurred when this process was carried out at temperatures lower than -5° C. Table III shows that the ether step increases the activity of the extracted enzyme.

TABLE II

The Effect of Mitochondrial pH on Acetone Powder ATPase Activity

Preparation	pH of Mitochondrial suspension before Acetone Dessication	ATPase Activity moles Pi formed/ mg. protein/30 min.
Whole Mitochondria	-----	1.64
Acetone Powders		
A	7.4	5.74
B	6.5	7.00
C	6.0	8.94
D	5.5	3.37

Each flask contained 30 μmoles ATP, pH 7.4; 300 μmoles tris-maleate buffer, pH 7.4; 15 μmoles $MgCl_2$; 1.0 ml. enzyme containing 1.0-1.2 mg. protein/ml. (Whole Mitochondria conc: 10.91 mg. protein/ml.) in a total volume of 3.0 ml.

Incubation was for 30 minutes at 30° C.

TABLE III

The Effect of Ether on Acetone Powder ATPase Activity

Preparation	Wash Medium	ATPase Activity moles Pi formed/ mg. protein/30 min.
1. Acetone powder		
A	Acetone	3.86
B	Acetone plus ether	4.67
2. Whole Mitochondria	--	1.66

The Incubation conditions are the same as described in Table II, except that the mitochondrial protein concentration was 7.28 mg./ml. pH of mitochondria before acetone dessication was 7.4.

N-Butanol Extraction. Morton (55, p. 1092-1095) discovered that the butyl alcohols, particularly the n-butyl and isobutyl isomeres, possess an unique ability to dissociate lipoprotein complexes without causing denaturation. The enzymes of mitochondrial membranes and crista are closely associated with lipid material. Acetone powders contain considerable amounts of acetone-insoluble phospholipid and tightly bound lipoprotein complexes, and it was found that extraction of the dried acetone powder from cabbage mitochondria with n-butanol at -18° C. significantly increased the activity of the soluble enzyme preparations, presumably through the removal of lipid or phospholipid bound to the protein. The results of n-butanol treatment on the acetone powder are shown in Table IV.

Extraction of ATPase Activity. In an attempt to find a suitable medium for the extraction of the enzyme, several aqueous salt solutions at various concentrations were tested under different conditions. The results are shown in Table V.

It is clear that extraction is favored by $KHCO_3$ over $Na_2P_2O_7$, $NaHCO_3$, water, or tris-maleate buffer. Extraction for more than 10 minutes did not result in a significant increase in enzyme activity, although preparations extracted with 0.05 M bicarbonate solution were used for routine extraction

TABLE IV

The Effect of N-Butanol Extraction ATPase Activity from Acetone Powder

Experiment	Preparation	Extraction Temperature °C.	Extraction Time	ATPase Activity moles Pi formed/ mg. protein/30 min.
1.	Whole Mitochondria	---	---	1.50
	Acetone Powder A	---	---	5.40
	"	-18°	24 hrs.	6.31
2.	Acetone Powder B	---	---	3.83
	"	-18°	15 min.	4.41
	"	"	12 hrs.	5.56
	"	"	24 hrs.	6.58
	"	"	48 hrs.	6.10
		24°	24 hrs.	3.48

Incubation conditions are the same as those described in Table II. Mitochondrial protein concentration was 12.8 mg./ml.

TABLE V

Comparison of Various Substances During ATPase Extraction from Acetone Powder

Exp. No.	Extraction Medium	ATPase Activity in Supernatant moles Pi formed/ mg. protein/30 min.
1.	water	4.86
	0.05 M $Na_2P_2O_7$	5.14
	0.05 M $KHCO_3$	6.11
2.	0.01 M $Na_2P_2O_7$	4.30
	0.05 M $Na_2P_2O_7$	5.14
	0.10 M $Na_2P_2O_7$	5.85
3.	water	4.86
	0.01 M $KHCO_3$	4.90
	0.05 M $KHCO_3$	6.24
	0.10 M $KHCO_3$	6.84
	0.2 M $KHCO_3$	6.41
	0.5 M $KHCO_3$	5.78
4.	0.05 M $KHCO_3$	6.11
	0.05 M $KHCO_3$ plus 5×10^{-3} M Mg^{++}	6.26
5.	0.01 M $KHCO_3$ 10 min. extraction	3.20
	0.01 M $KHCO_3$ 60 min. extraction	3.32
6.	water	2.84
	0.01 M $KHCO_3$	5.67
	0.01 M $NaHCO_3$	5.36
	0.05 M tris-maleate buffer, pH 7.4	4.20

The incubation conditions are the same as described in Table II.

of the acetone powder rather than higher concentrations for two reasons: first, it was important to keep the ionic strength of the extract as low as possible in order to facilitate rapid salt removal with Sephadex chromatography before fractionation on DEAE-cellulose, and secondly, the increase in activity obtained by employing $KHCO_3$ at a higher ionic strength was not significantly large.

It was found that successive re-extractions of the acetone powder residue were useful in recovering more enzyme. Essentially all of the extractable ATPase activity could be removed after three extractions.

Part III

Further Purification of the ATPase Activity

A wide variety of enzyme fractionation techniques were employed in an attempt to further purify the solubilized ATPase activity. Purifications exceeding 3-fold from the solubilized extracts of either source-- the sonicated mitochondrial fraction or the acetone powder--were not achieved. Thus the maximum purification of ATPase activity above that for whole mitochondria was approximately 15-fold. The results of various purification procedures are shown in Table VI.

TABLE VI

Summary of Procedures Attempted during ATPase Purification Study

Purification Technique employed	Source of soluble enzyme	Purification Factor	Approximate yield of purified enzyme
Ammonium sulfate fractionation	sonicated cabbage mitochondria	3-fold	10%
Alcohol fractionation at -15° C.	" "	none	---
Acetone fractionation at -15° C.	Acetone Powder	2-fold	12%
Temperature fractionation	both	none	---
Isoelectric Precipitation	both	none	---
Chromatography by Sephadex G-75	Acetone Powder	2-fold	15%
Chromatography by DEAE cellulose	" "	3-fold	25%

TABLE VI Cont.

Purification Technique employed	Source of soluble enzyme	Purification Factor	Approximate yield of purified enzyme
Chromatography by carboxy methyl cellulose	Acetone Powder	none	---
Chromatography by DEAE-Sephadex	" "	none	---
Chromatography by Calcium Phosphate Gel	" "	none	---
Protamine fractionation	" "	none	---

The purification factors shown are those obtained from soluble enzyme fractions derived from either sonic-treated mitochondria or from mitochondrial acetone as indicated.

Purification from the soluble Fraction of Sonic-treated Mitochondria. Table VII illustrates a typical enzyme preparation from sonicated cabbage mitochondria. The final yield in this particular preparation was somewhat lower than that usually obtained, which varied between 12% and 14%.

Previous attempts at purifying the ATPase activity from the soluble fraction of sonic-comminuted cabbage mitochondria by ammonium sulfate fractionation were unsuccessful. The final procedure was arrived at after a number of experiments had disclosed that the enzyme activity could be markedly stabilized in aqueous solution at pH 6.0 in the presence of a sulfhydryl reagent at 1×10^{-3} M ATP.

Purification from Soluble Acetone Powder Extracts. The recent success of DEAE-cellulose in protein purification made this material a logical choice for attempts at eliciting a further purification of the ATPase from acetone powder extracts. The results are summarized in Table VIII. A preliminary study indicated that the pH of maximum protein adsorbtion was near pH 8.0 as shown in Figure 2. The capacity of the DEAE-cellulose at pH 8.2 for the ATPase activity was determined and found to be approximately 0.4 mg. enzyme protein/mg. cellulose. The results of this study are shown in

TABLE VII

Summary of Purification Procedure employed with Soluble Fraction of sonic-treated Cabbage Mitochondria

Steps During Purification	Volume ml.	Total protein mg.	TOTAL UNITS soluble	TOTAL UNITS particulate	Specific Activity units/mg. protein	Yield %
Whole Mitochondria	100	820	---	742	0.91	100
After 45 minutes sonication followed by centrifugation at 105,000 x g	92	332	2040	1480	6.15	40.5
After re-extraction	15	29	114	---	1.80	---
Combined Soluble Fractions	107	354	2154	---	6.10	43.2
40-55% ammonium sulfate fraction	25	38	518	---	13.8	8.7

A unit of activity represents moles of Pi formed/mg. enzyme protein/30 minute incubation at 30° C. in a 2.0 ml. of a medium consisting of 20 μmoles ATP, pH 7.4; 200 μmoles tris-acetate buffer, pH 7.4; 10 μmoles $MgCl_2$; and 1.0 ml. enzyme containing 1.2-1.5 mg. protein/ml.

TABLE VIII

Summary of ATPase Purification Procedure Employed with soluble fraction of Cabbage Mitochondrial Acetone Powder

Steps During Purification	Volume ml.	Total protein mg.	Total Units	Specific Activity units/mg.protein	Yield
N-Butanol Extracted Crude Acetone Powder Extract	80	415	2000	5.0	100%
After Sephadex Concentration and Buffer Exchange	50	247	1500	6.1	60%
After DEAE Cellulose Column Chromatography	40	120	1200	10.0	29%

A unit of activity is the same as defined in Table VII.

Figure 3.

A typical elution diagram obtained during DEAE-cellulose chromatography of aqueous acetone powder extracts is shown in Figure 4. The protein solution was found to migrate down the column in an initial amber-colored band, becoming separated into several distinct regions as the elution progressed. It is interesting that one of the bands possessed an unusual blue color that eventually disappeared and was replaced by a less intense yellow band that left the column at the same time that the ATPase activity began to appear in the eluant fractions.

The appearance of the blue band during the chromatography cannot be explained at the present time and its significance must await further work.

It was found, as mentioned earlier, that the protein fractions associated with the ATPase activity possessed a characteristic yellow coloration that was non-dialyzable and could not be removed by acid precipitation. It was further found that the enzyme solutions exhibiting ATPase activity and the characteristic yellow coloration also contained a non-dialyzable, acid-labile source of inorganic phosphate. The relationship of this acid-labile phosphate to the ATPase

activity and protein peaks in the eluant fractions is shown in Figure 4. The shaded area represents the phosphate formed after brief treatment of the dialyzed enzyme solutions with 4N H_2SO_4. It should be noted that the peaks representing acid-labile phosphate, ATPase activity, and protein are essentially coincident. Furthermore, no acid-labile inorganic phosphate was detected in any of the dialyzed protein solutions devoid of ATPase activity.

These results, although inconclusive at the present time represent only preliminary experimental findings but nevertheless suggest that a possible intermediate in oxidative phosphorylation has been isolated from aqueous extracts of acetone-dessicated cabbage mitochondria. The proposed intermediate could conceivably be a phospho-protein complex, similar to the one proposed by Boyer, et al. (72, p. 43-47) and would be represented by P~C in Equations (3) and (4) presented in the "Introduction".

Support for the existence of such an intermediate in oxidative phosphorylation has recently been put forth by Boyer and co-workers (72, p. 43-47). Further investigations of these results must be carried out before the actual significance of these findings can be accessed. However, the fact

that the acid-labile phosphate can be detected in significant amounts only in the dialyzed protein solutions associated with the ATPase activity is particularly interesting in connection with the findings of Pullman, et al. (64, p. 3322-3329) and Racker (65, p. 54) that a soluble ATPase from beef-heart mitochondria was required for oxidative phosphorylation and furthermore, that is also possessed an activity catalyzing the coupled synthesis of ATP from ADP and inorganic phosphate.

It would be premature at this point to suggest an analogous role for the ATPase from cabbage mitochondria but the possibility of such a relationship between ATPase activity and oxidative phosphorylation in cabbage mitochondria should be investigated.

Part IV

Characterization of the ATPase Activity

The recovery and distribution of the ATPase activity have been described earlier. For the characterization studies it was assumed that the same enzyme preparation could be obtained from either procedure-- the chromatographic purification from acetone powder or the ammonium sulfate fractionation of sonic-treated whole mitochondria.

FIGURE 2

The Effect of pH on the Capacity of DEAE-Cellulose for ATPase Activity

Each flask contained 20 μmoles ATP, pH 7.2; 15 μmoles $MgCl_2$; 100 μmoles tris-maleate buffer and 1.0 ml. enzyme solution containing 3.70 mg. protein/ml. Incubation was for 30 minutes at 30° C.

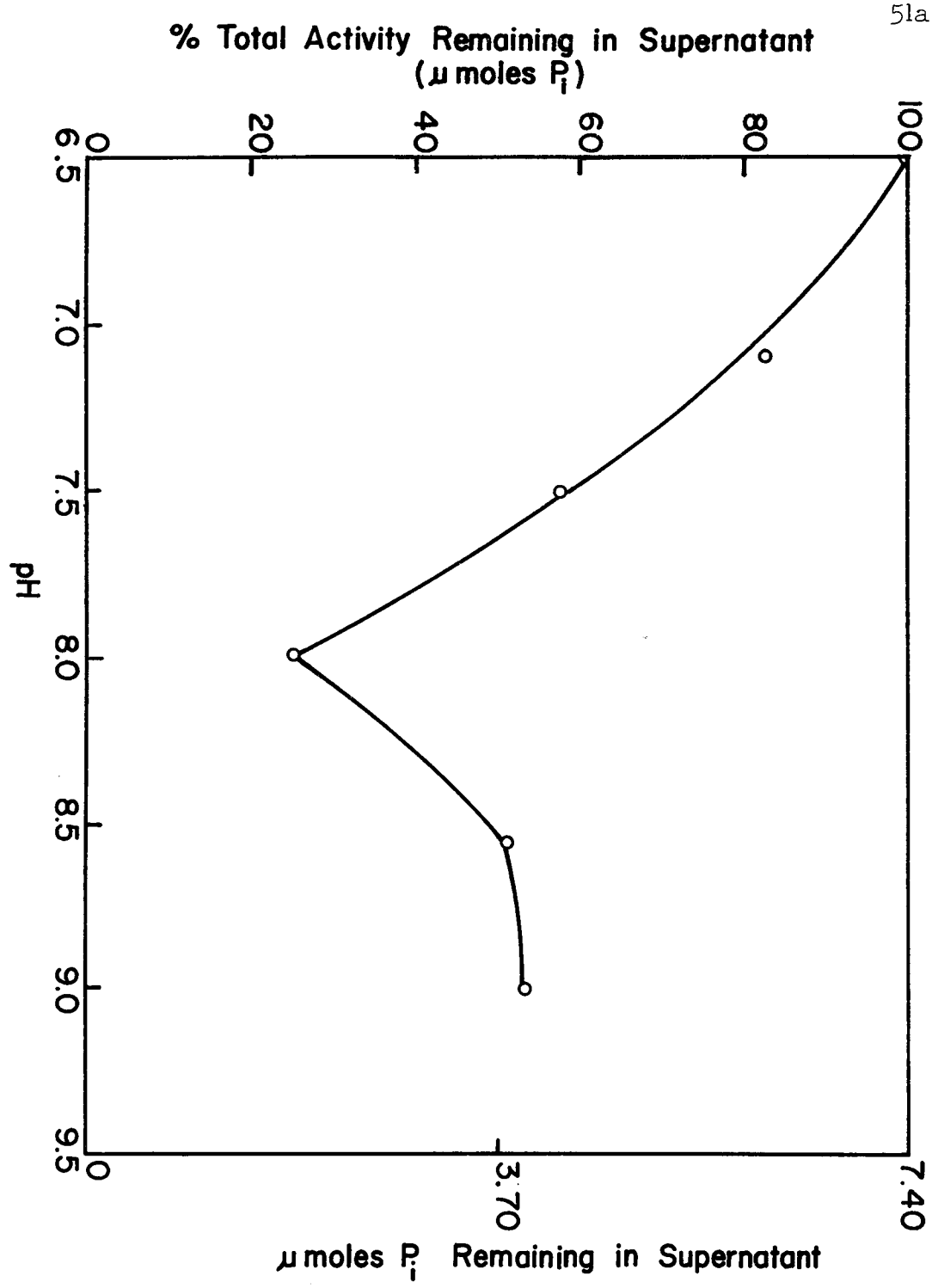
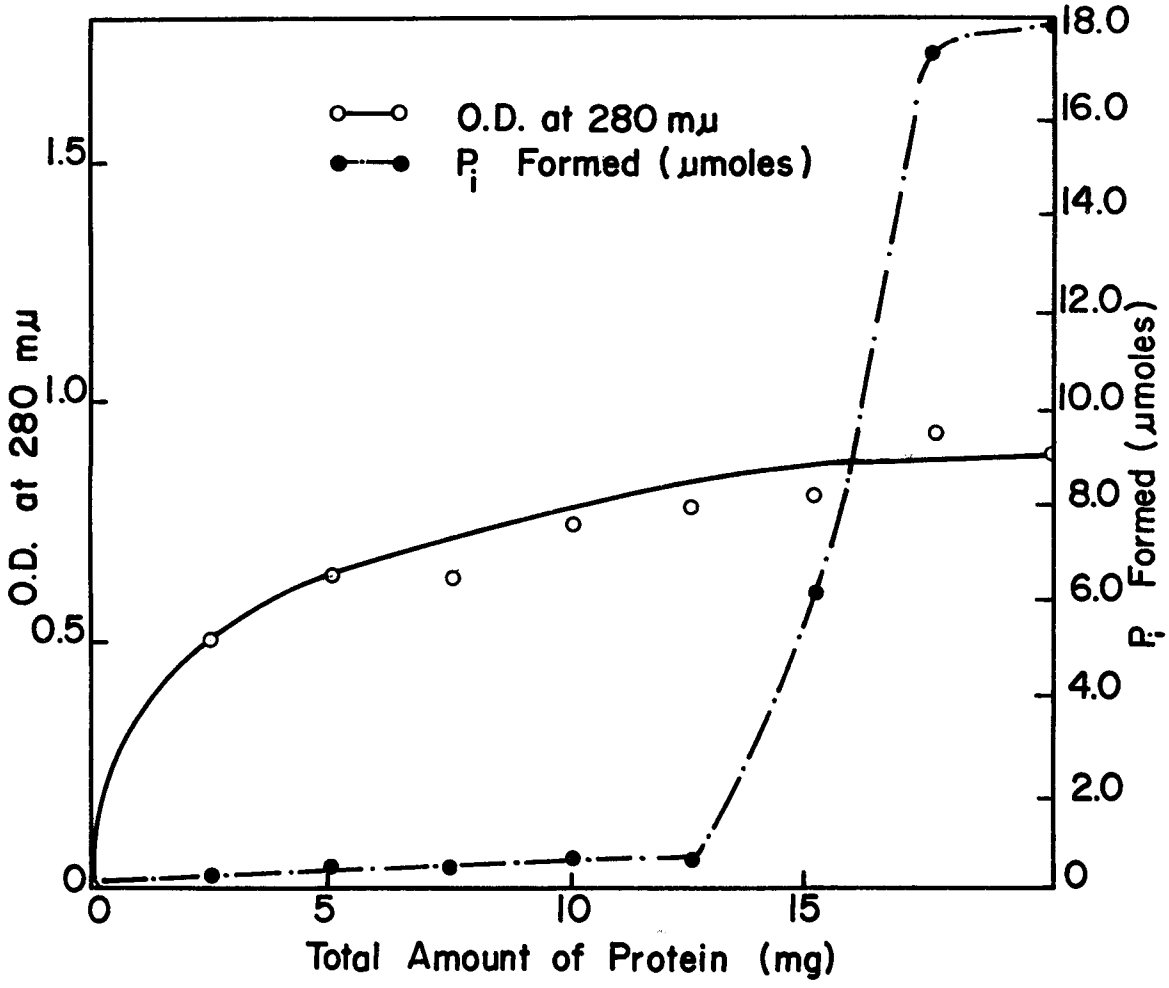

% Total Activity Remaining in Supernatant
(μ moles P_i)
0
20
40
60
80
100
pH
6.5
7.0
7.5
8.0
8.5
9.0
9.5
0
3.70
7.40
μ moles P_i Remaining in Supernatant

FIGURE 2

FIGURE 3

The Capacity of DEAE-Cellulose for ATPase Activity

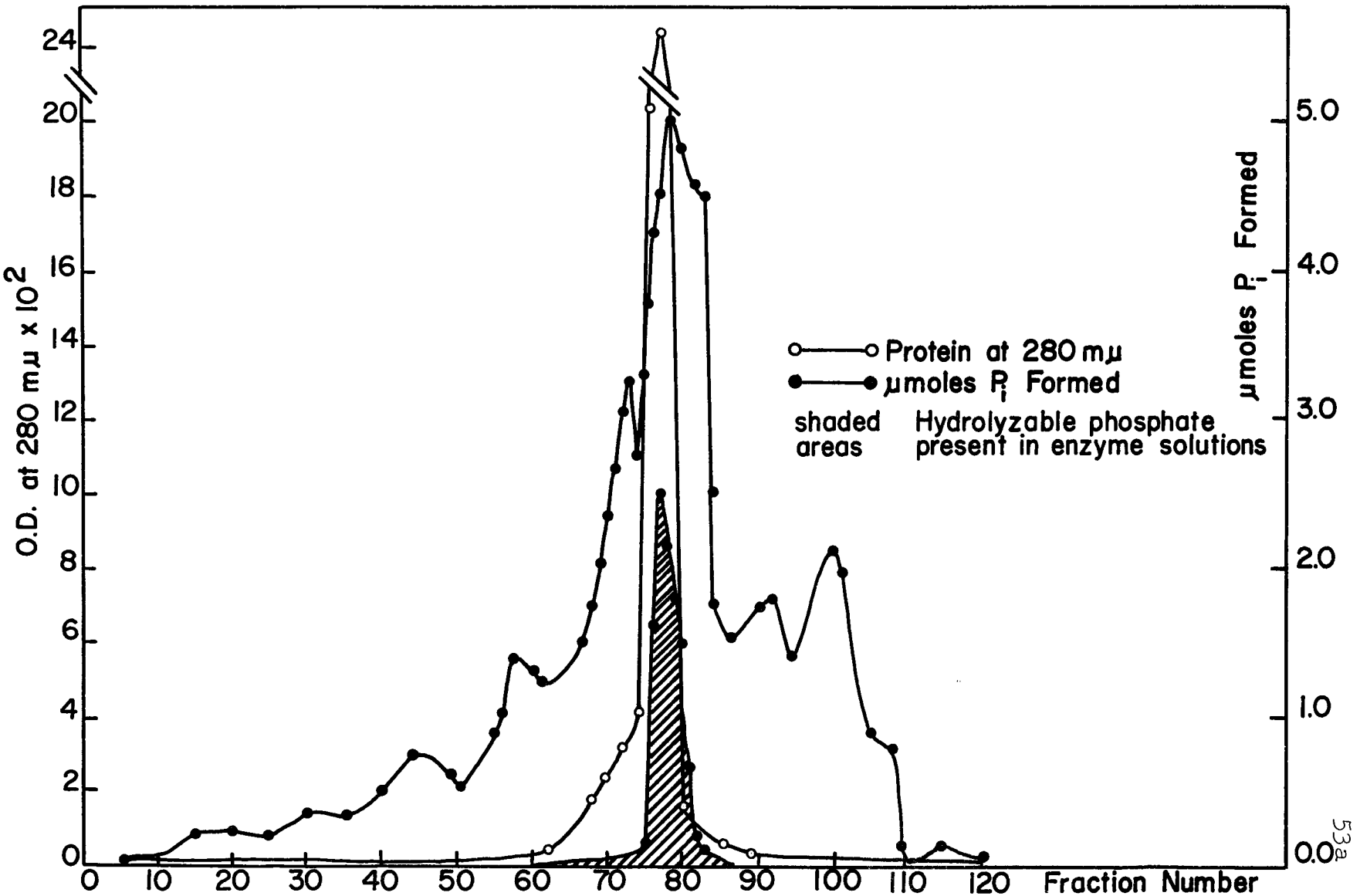


Incubation conditions are the same as those described in Figure 2.

FIGURE 4

Purification of the ATPase Activity by DEAE-Cellulose Chromatography

Eluant fractions were assayed with the same systems as described in Figure 2. For the demonstration of inorganic phosphate (shaded area) protein solutions were dialyzed 8 hours against 0.01 M tris-HCl buffer, pH 7.4, and then assayed directly for inorganic phosphate.


O.D. at 280 mμ x 10²
μmoles Pi Formed
Protein at 280 mμ
μmoles Pi Formed
shaded areas Hydrolyzable phosphate present in enzyme solutions
Fraction Number
0
10
20
30
40
50
60
70
80
90
100
110
120
2
4
6
8
10
12
14
16
18
20
24
1.0
2.0
3.0
4.0
5.0

Effect of pH. A detailed study of the ATPase activity was carried out in a number of different buffer systems including tris-maleate, collodine-HCl, veronal-HCl, tris-acetate-NH_3 and glycyl-glycine-histidine buffers from pH 5.5 to 9.0. Veronal-HCl buffer was found to be inhibitory to the enzyme in the pH range studied. The effect of pH on ATPase activity in three buffer systems is shown in Figure 5.

It was observed in preliminary studies on the behavior of the ATPase with varying pH that the crude supernatant fraction from sonicated mitochondria contained at least two and possibly three pH optima. Two minor optima were found at pH 6.6-6.7 and pH 7.7-7.8, while the major activity was found to be at pH 7.1-7.2. In numerous experiments on the 15-fold purified ATPase, these three rather characteristic pH optima could be demonstrated. The concept of multiple ATPases in mitochondria is consistent with the belief that oxidative phosphorylation occurs at three sites along the respiratory chain. Thus Slater (70, p. 288-294) has predicted that mitochondria contain three different DNP-sensitive enzyme systems which may effect the hydrolysis of ATP. Meyers and Slater (53, p. 363-364) studied the pH-activity curves of liver mitochondria and showed the presence of four different

FIGURE 5

The Effect of pH on ATPase Activity in Different Buffer Systems

Each flask contained 20 μmoles ATP; 200 μmoles of the indicated buffer at the pH shown; 15 μmoles $MgCl_2$; and 1.0 ml. enzyme solution containing 0.9-1.0 mg. protein/ml. in a final volume of 2.0 ml. Incubation time was 20 minutes at 30° C. The results are the average of duplicate flasks.

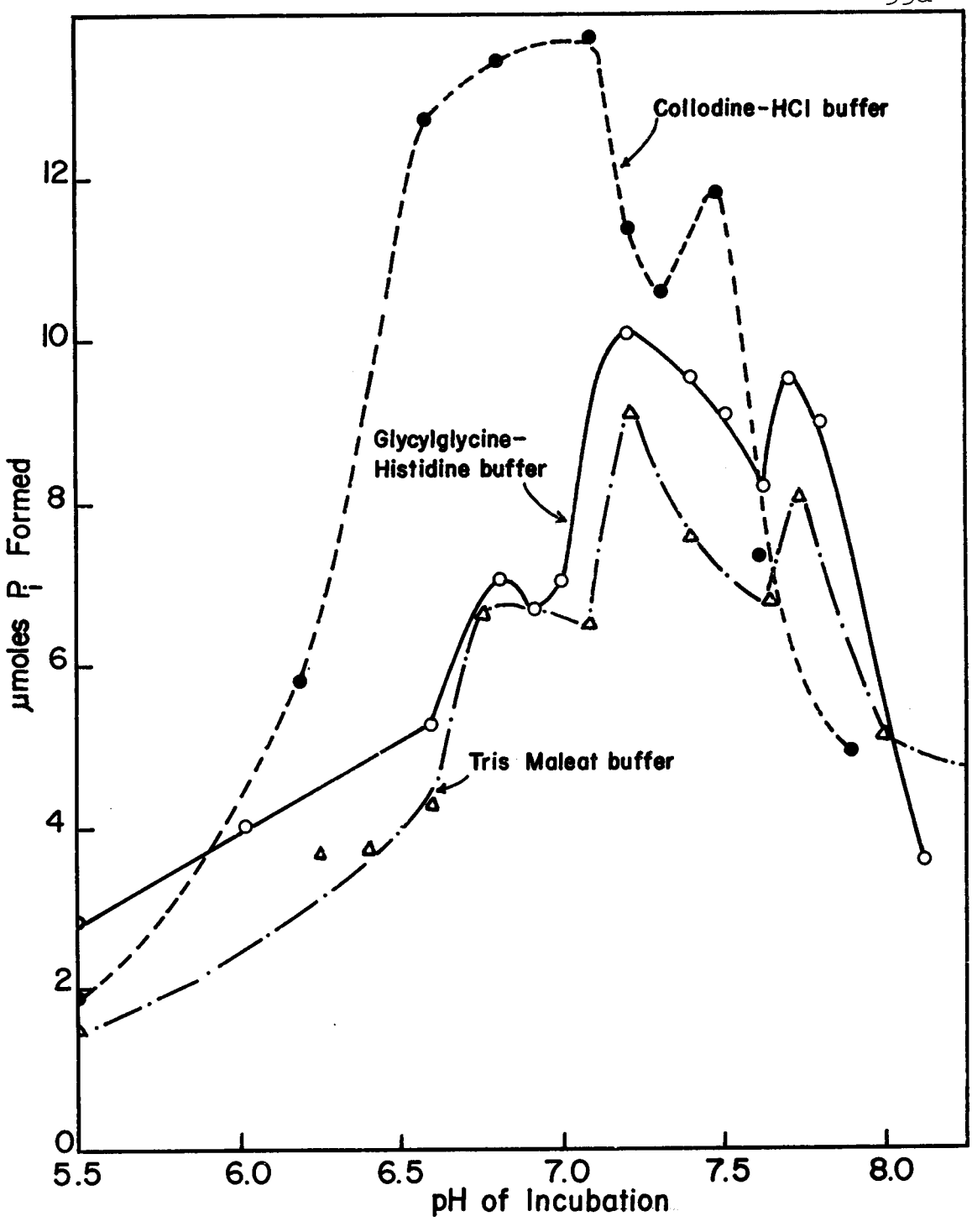


FIGURE 5

The Effect of pH on ATPase Activity in Different Buffer Systems

peaks of activity. Of these four pH optima, three were stimulated by DNP. It was thus postulated by Slater that, depending on the substrate used, the number of peaks in the pH-activity curve of oxidative phosphorylation is equal to the number of phosphorylation sites along the respiratory chain, and that these pH values are identical with the observed ATPase pH optima. Aloni and Poljakoff-Mayber (3, p. 105-110) found at least two and probably three enzymes possessing ATPase activity in mitochondria isolated from lettuce seedlings. These workers found that most of the ATPase activity could be concentrated in the soluble fraction, in agreement with the soluble ATPase studies by Young and Varner (75, p. 71-78) with Alaska peas and with the present study on the soluble ATPase from cabbage mitochondria.

With respect to the nature of the pH curves described in the present study, it would be premature to speculate on any relationship which might possibly exist between the varying pH maxima and oxidative phosphorylation. Further studies on a more highly purified enzyme are needed in order to resolve the many questions that cannot be answered at present.

Effect of Oleic Acid. It is well known that certain long chain free fatty acids such as myristic acid, palmitic acid and

stearic acid and cis-isomers of unsaturated fatty acids such as oleic acid stimulate the ATPase in rat liver mitochondria (63, p. 458-466).

Oleic acid at 5×10^{-3} M was found to effect a 10-15% stimulation of the ATPase activity in the pH range 5.5 to 8.0. At higher pH values the presence of oleic acid was inhibitory, due in part, perhaps, to the increased proportion of oleic acid present in the form of a salt. Oleic acid may therefore have to exist primarily in its free state in order to elicit maximum ATPase stimulation. The effect of oleic acid on the ATPase activity is shown in Figure 6.

Oleic acid has been widely employed in the study of mammalian mitochondrial systems since it has been found to uncouple oxidative phosphorylation, stimulate ATPase activity, inhibit the ATP-P^{32} exchange reaction and release respiration (42, p. 2459-2464). These are precisely the same effects elicited by DNP in mammalian mitochondrial preparations and this may indicate that DNP and oleic acid act at the same point on the respiratory chain in mammalian mitochondria. Lotlikar (45, p. 70-78) showed that oleic acid at a concentration of 1×10^{-3} M and 3×10^{-3} M stimulated the ATPase activity of intact cabbage mitochondria 3-fold and 4-fold,

FIGURE 6

The Effect of Oleic Acid on ATPase Activity

Each flask contained 20 μmoles ATP; 200 μmoles tris-acetate - NH_3 buffer at the indicated pH; 15 μmoles $MgCl_2$; 1.0 ml. enzyme solution containing 0.9-1.0 mg. protein/ ml.; and, where present, 15 μmoles oleic acid in a total volume of 2.0 ml. Incubation time was 20 minutes at 30° C. The results are the average of duplicate flasks.

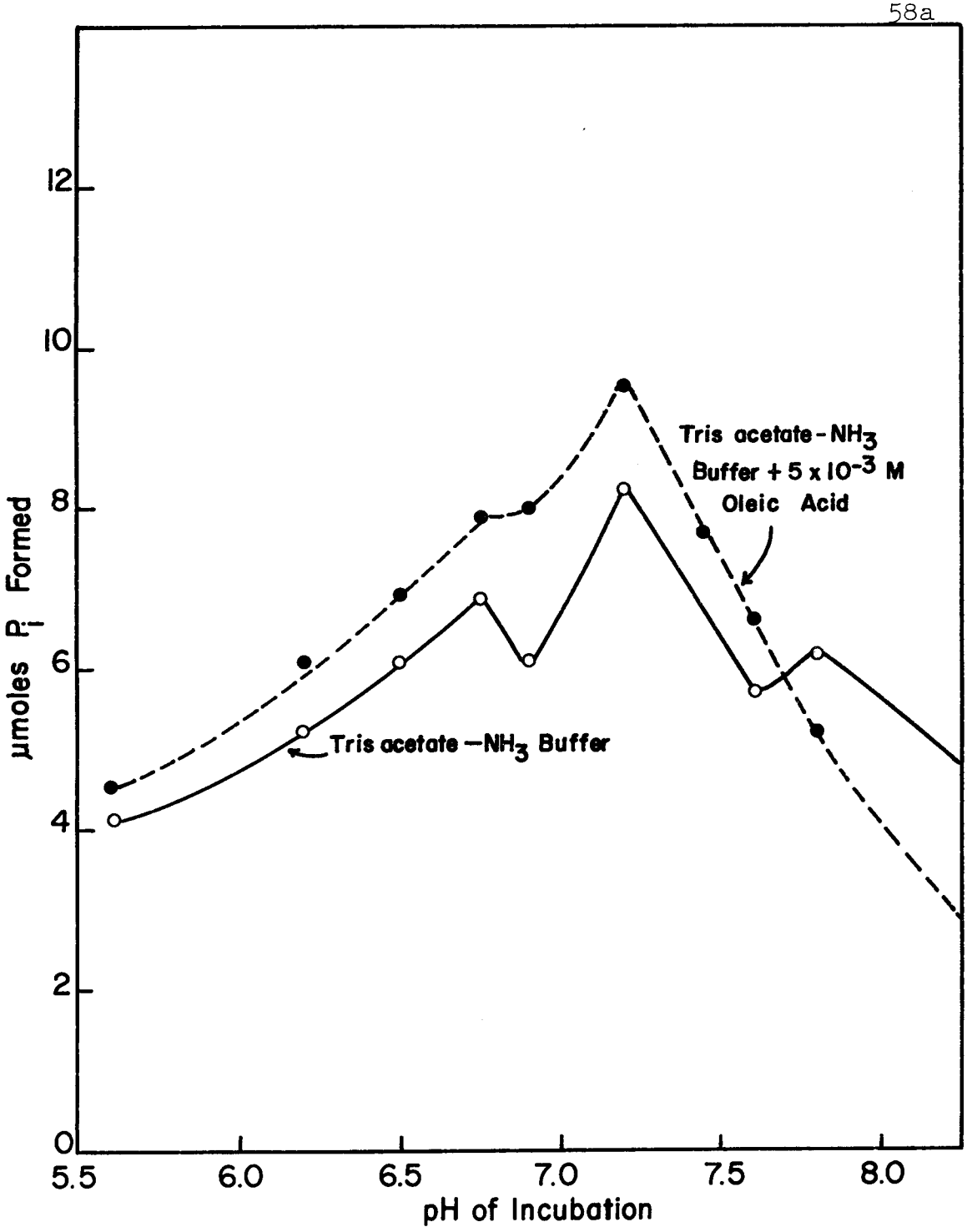


FIGURE 6

The Effect of Oleic Acid on ATPase Activity

respectively, but only in the presence of added Mg^{++}. In the present studies, oleic acid stimulated the purified ATPase in the three buffer systems used. The effect of oleic acid on the ATPase at various pH values was not studied in the absence of added Mg^{++} since it was shown by Lotlikar that intact cabbage mitochondrial ATPase was dependent on added Mg^{++} for maximum stimulation by oleic acid. Lotlikar also showed in detailed studies on cabbage mitochondrial phosphatase activity with adenosinemonophosphate (AMP), ADP and ATP as substrates that the main action of oleic acid was to stimulate the hydrolysis of ATP and not to stimulate the activity of an apyrase activity (an enzyme which catalyzes the hydrolysis of ATP to AMP and orthophosphate) shown by Mazelis (48, p. 153-158) to be present in the cytoplasmic fraction of cabbage.

The Effect of DNP. DNP and other uncoupling agents have been shown to stimulate the ATPase activity in many mammalian mitochondrial systems, but there has been only one case where DNP has been shown to stimulate plant mitochondria (20, p. 898-909). Lotlikar (45, p. 51-62) was unable to achieve DNP stimulation of intact cabbage mitochondrial ATPase and suggested that this may be a fundamental difference

between plant and animal mitochondria. It was found in the present studies that DNP at 5 x 10^{-4} M stimulated the purified ATPase over the pH range 5.5 to 9.0. These results are shown in Figure 7. It is possible that the mitochondrial membrane may have an important function in regulating the passage of cellular constituents in and out of the mitochondrion. Thus, the observed stimulation of the purified soluble cabbage mitochondrial ATPase activity by DNP and its absence in intact mitochondria might be explained in terms of a membrane effect, whereby the DNP is unable to penetrate the mitochondrial wall and come in contact with the ATPase. Since, however, DNP has been shown to stimulate animal mitochondrial ATPase, a fundamental difference between plant and animal mitochondria may express itself in terms of selective permeability towards various exogenous substances.

Requirement for Mg^{++}. Magnesium appears to be an obligatory requirement for enzyme catalyzed reactions involving ATP. Other metals such as Mn^{++} and Ca^{++} may substitute, in some cases, for Mg^{++}, but the activity may equal, but never exceed, that elicited by Mg^{++} alone. The purified ATPase from cabbage mitochondria was found to require Mg^{++} for maximum activity. The results are shown in

FIGURE 7

The Effect of DNP on ATPase Activity

The incubation conditions are the same as those described in Figure 6, except that, where indicated, DNP is substituted for oleic acid.

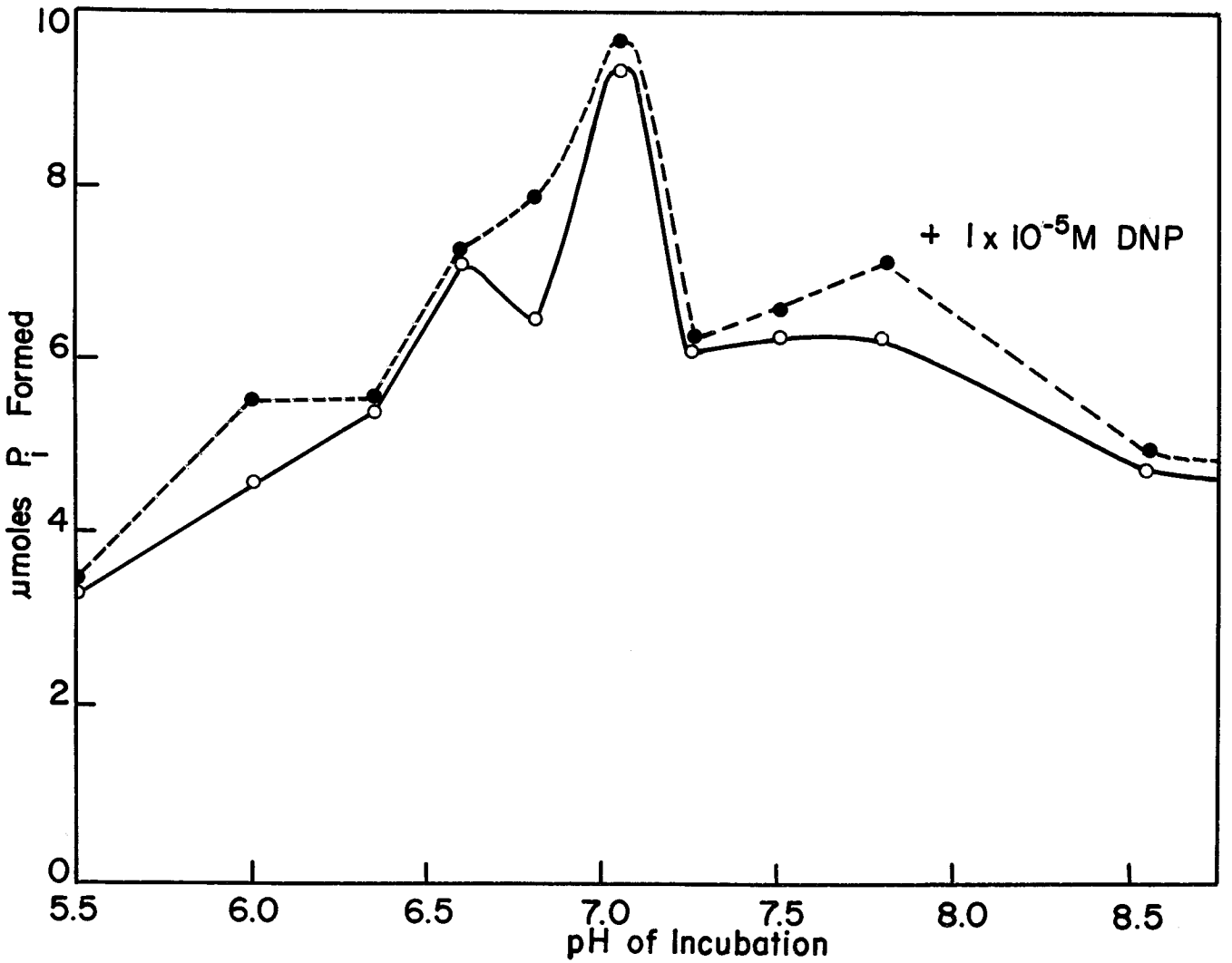

10
8
6
4
2
0
µmoles P_i Formed
+ 1 x 10^{-5}M DNP
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH of Incubation

FIGURE 7

Figure 8. Mg^{++} at a concentration of 5×10^{-3} M was found to give maximum stimulation. Higher concentrations of Mg^{++} became slightly inhibitory.

The necessary role of Mg^{++} in oxidative phosphorylation was demonstrated by Kielley and Bronk (34, p. 448-449) and later by Linnane and Ziegler (44, p. 630-638) and Linnane (43, p. 221-222) who showed that Mg^{++} was required in mitochondria as a chelating agent to bind a soluble component required for phosphorylation to the structure of the mitochondrion. The highly purified soluble ATPase of Pullman et al. (64, p. 3322-3329) has a remarkable similarity to the "soluble component" described above and since this ATPase possesses a coupling activity (i.e. an activity that "couples" the synthesis of ATP from ADP and Pi) inseperable from the ATPase activity, it becomes evident that there may be a close interrelationship between the Mg^{++} stimulated ATPase and oxidative phosphorylation.

Studies on other purified ATPases have shown that there is a certain Mg^{++}/ATP ratio that is required for maximum enzymic activity. Thus, the highly purified soluble ATPase from rat muscle (4, p. 255-261) was shown to have maximum activity when the ATP/Mg^{++} ratio was 4:1 of ATP

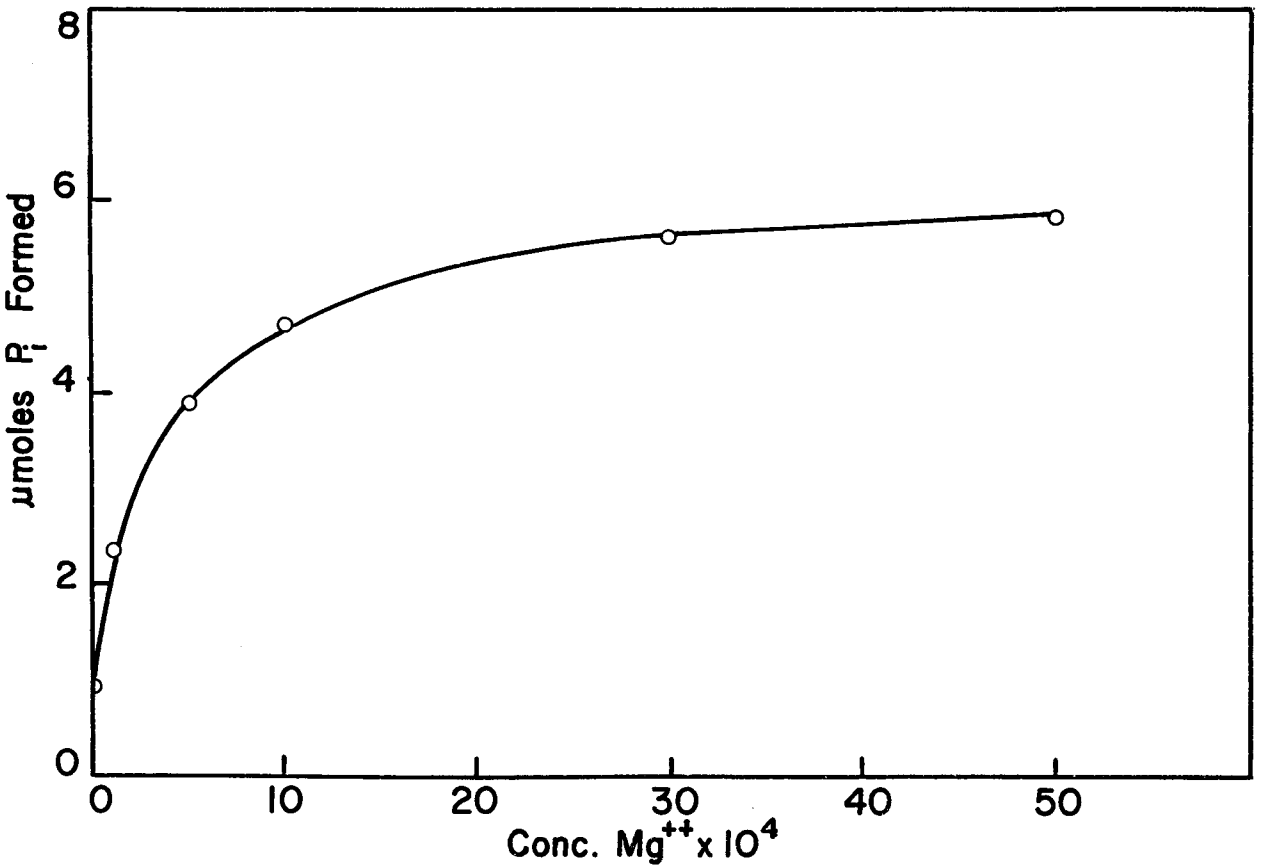


FIGURE 8

The Effect of Mg^{++} Concentration on ATPase Activity

Each flask contained 20 moles ATP, pH 7.2; 200 μmoles tris-acetate buffer, pH 7.2; the indicated amount of Mg^{++} (as $MgCl_2$) and 1.0 ml. enzyme solution containing 0.9-1.0 mg. protein/ml. in a total volume of 2.0 ml. Incubation time was 20 minutes at 30°C. The results are the average of duplicate flasks.

to Mg^{++}. The particulate ATPase purified by Kielley and Meyerhof (35, p. 591-601) exhibited maximum activity when the ATP/Mg^{++} ratio was 2:1. The optimum ATP/Mg^{++} ratio for the purified ATPase from cabbage mitochonfria, in contrast to the above, was determined and found to be 1:1. The results of this study are shown in Table IX.

It is difficult to formulate an explanation for the wide difference in Mg^{++} requirement between the animal and plant ATPase activities cited above. The value of the ATP/Mg^{++} ratio may reflect the number of catalytic sites on the protein taking part in the Mg^{++}-dependent enzymatic hydrolysis of ATP but such speculation should be supported by more experimental evidence.

Absorbtion Spectrum. The ATPase activity of the purified protein was always found to be associated with an intense, non-dialyzable yellow color. A spectrophotometric scan of the enzyme in both an oxidized and reduced (by sodium dithionite) state from 670 mμ to 280mμ gave no evidence of a flavin moiety associated with the enzyme. Mandeles (46, p. 256-264) observed a similar intense yellow coloration associated with a single protein peak obtained during DEAE-cellulose chromatography of an undialyzed extract prepared

TABLE IX

The Effect of Varying the ATP/Mg^{++} Ratio on ATPase Activity

Conc. Mg^{++}	Ratio, ATP/Mg^{++}	μmoles Pi formed
0	none	0.84
2.5×10^{-3}	4:1	6.40
3.3×10^{-3}	3:1	7.22
5.0×10^{-3}	2:1	7.70
1.0×10^{-2}	1:1	12.10
2.0×10^{-2}	1:2	8.91
3.0×10^{-2}	1:3	5.78
4.0×10^{-2}	1:4	4.33

Each flask contained 20 μmoles ATP, pH 7.2; 200 μmoles tris-Acetate Buffer, pH 7.2; the indicated amount of Mg^{++} and 1.0 ml. enzyme solution containing 0.9-1.0 mg. protein/ml. in a total volume of 2.0 ml.

Incubation was for 20 minutes at 30° C.

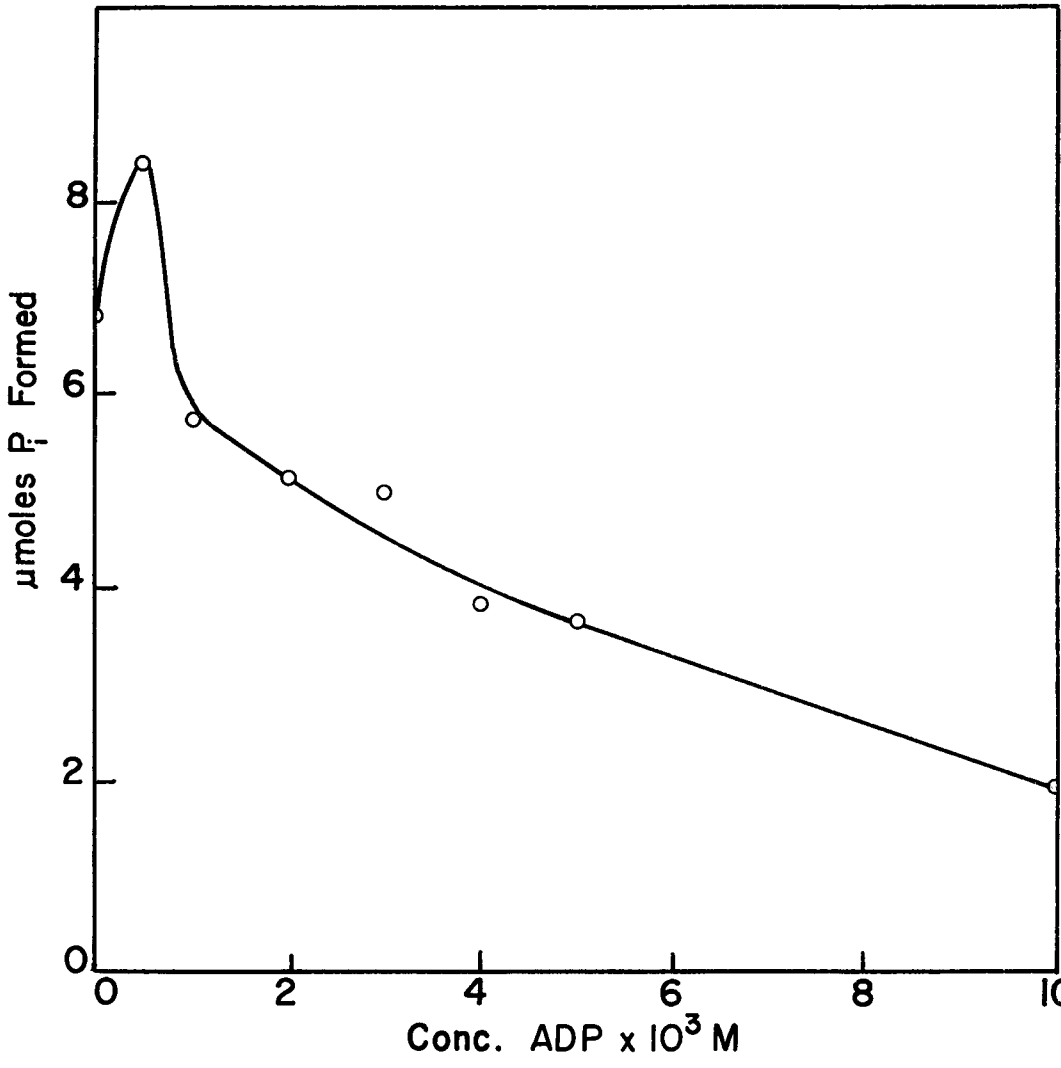
from a fresh cabbage homogenate. The peak was found to have apyrase, inorganic pyrophosphatase, and acid phosphatase activity. There was no evidence found indicating the presence of flavin.

Penniall (57, p. 6195-6196) has indicated the possible involvement of flavin with a soluble ATPase from acetone dessicated rat-liver mitochondria. Although these results provide the first direct evidence for the possible participation of a flavin (FAD) in the ATPase activity associated with mammalian systems, they are at variance with the results of the present study.

Effect of ADP. ADP has been shown to be a powerful inhibitor of ATPase activity in rat liver mitochondria (36, p. 213-222; 23, p. 1015-1023) and in plant mitochondria (20, p. 898-909). It was suggested by Kielley and Kielley (36, p. 213-222) that ADP may compete for Mg^{++} with ATP and therefore result in competitive inhibition. The ATPase from cabbage mitochondria was found to be inhibited by ADP at concentrations greater than 5×10^{-4} M. The results are shown in Figure 9. It will be noted that at 5×10^{-4} M ADP there is a slight stimulation of the ATPase activity but that the enzyme becomes rapidly inhibited at higher

FIGURE 9

The Effect of ADP Concentration on ATPase Activity



Each flask contained 20 µmoles ATP, pH 7.2; 200 µmoles tris-acetate buffer, pH 7.2; 15 µmoles $MgCl_2$; 1.0 ml. enzyme containing 0.9-1.0 mg. protein/ml. and, where indicated, the designated amounts of ADP. Final volume was 2.0 ml. Incubation time was 30 minutes at 30° C. The results are the average of duplicate flasks.

concentrations. This indicates that the ATPase can tolerate a low (5×10^{-4} M) concentration of ADP and that there is evidently some contaminating phosphatase activity present that hydrolyzes ADP to AMP + Pi, resulting in the apparent ATPase stimulation.

Effect of 2,4-D. The herbicide 2,4-D is known to inhibit oxidative phosphorylation in plant mitochondria and the exact locus of its action has been extensively studied by Lotlikar (45, p. 1-99). Since the Mg^{++}-stimulated ATPase may represent a part of the oxidative phosphorylation mechanism described in the "Introduction", it would be of interest to determine what effect 2,4-D has on the Mg^{++}-stimulated ATPase. Lotlikar (45, p. 65-78) found that 2,4-D at concentrations 1×10^{-4} M to 1×10^{-2} M had no effect on the Mg^{++}-stimulated ATPase of cabbage mitochondria. The effect of varying concentrations of 2,4-D on the purified ATPase are shown in Table X. Low concentrations of 2,4-D (1×10^{-6} M to 1×10^{-5} M) were found to stimulate the ATPase activity slightly, whereas concentrations below 1×10^{-5} M resulted in inhibition. 2,4-D at 5×10^{-6} M resulted in maximum stimulation (26%) above that for complete absence of 2,4-D. These results are different than those obtained by Lotlikar,

TABLE X

The Effect of 2,4-D on ATPase Activity

Concentration of 2,4-D	μmoles Pi formed
none	8.42
1×10^{-6} M	9.72
5×10^{-6} M	10.60
1×10^{-5} M	9.55
5×10^{-5} M	7.80
1×10^{-4} M	5.82

The incubation conditions are the same as described in Table IX except that incubation time was 30 minutes and Mg^{++} was present in all flasks at 5×10^{-3} M.

since he observed no ATPase inhibition with concentrations of 2,4-D as high as 1×10^{-2} M.

The evidence gathered from 2,4-D data points to a major difference between the ATPase system studied by Lotlikar and the one described in the present study. This difference may be explained, as discussed earlier, as arising from a change in the "availability" of active sites located on the enzyme protein. While bound to the intact mitochondrion, the ATPase would be essentially unaffected by the presence of 2,4-D, since there would conceivably be a "masking" of active sites on the enzymes as a consequence of close protein-protein interaction or steric factors. On the other hand, enzyme liberated from such confinement would be fully vulnerable to the action of inhibitory (or stimulatory) concentrations of 2,4-D.

Effect of Incubation Time. The presence of the ATP regenerating system is required for a continued linear relationship between enzyme activity and time as shown in Figure 10. It can be seen that in the absence of the ATP regenerating system the time-activity plot begins to deviate from linearity much earlier than in its presence. It is also evident that the relative rate of stimulation by oleic acid is considerably

FIGURE 10

The Effect of Incubation Time on ATPase Activity

Each flask contained 10 μmoles ATP, pH 7.2; 200 μmoles tris-acetate buffer, pH 7.2; 7.5 μmoles $MgCl_2$; 1.0 ml. of enzyme solution containing 0.9-1.0 mg. protein/ml. and, when present, 1 x 10^{-3} M oleic acid; 5 μmoles phosphoenolpyruvate, pH 7.2; and 32 μg pyruvic kinase in a final volume of 2.0 ml. Incubation time was 20 minutes at 30° C. The results are the average of duplicate flasks. (0——0) Indicate the presence and (●----●) the absence of an ATP regenerating system.

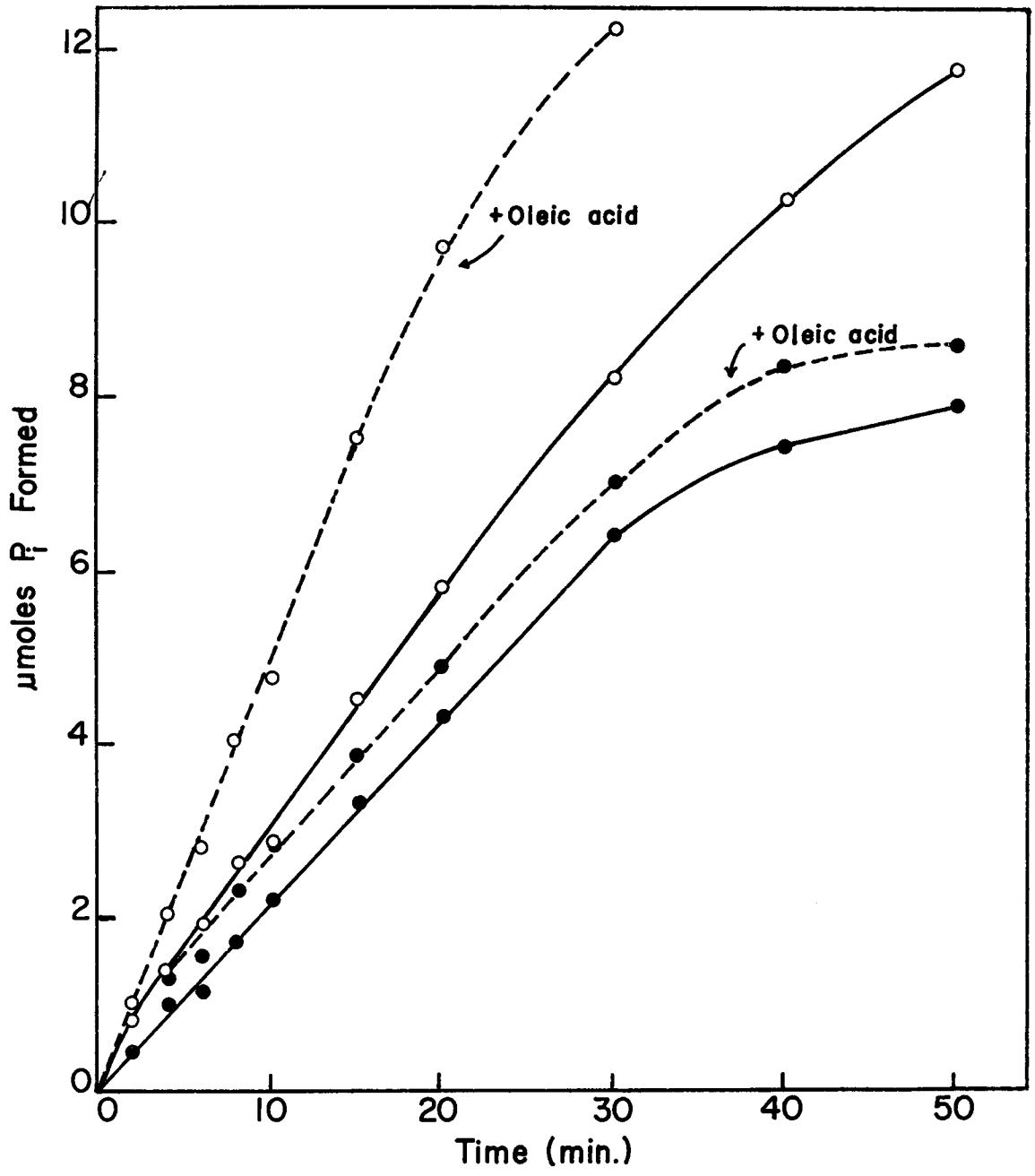

12
10
8
6
4
2
0
µmoles P_i Formed
+Oleic acid
+Oleic acid
0
10
20
30
40
50
Time (min.)

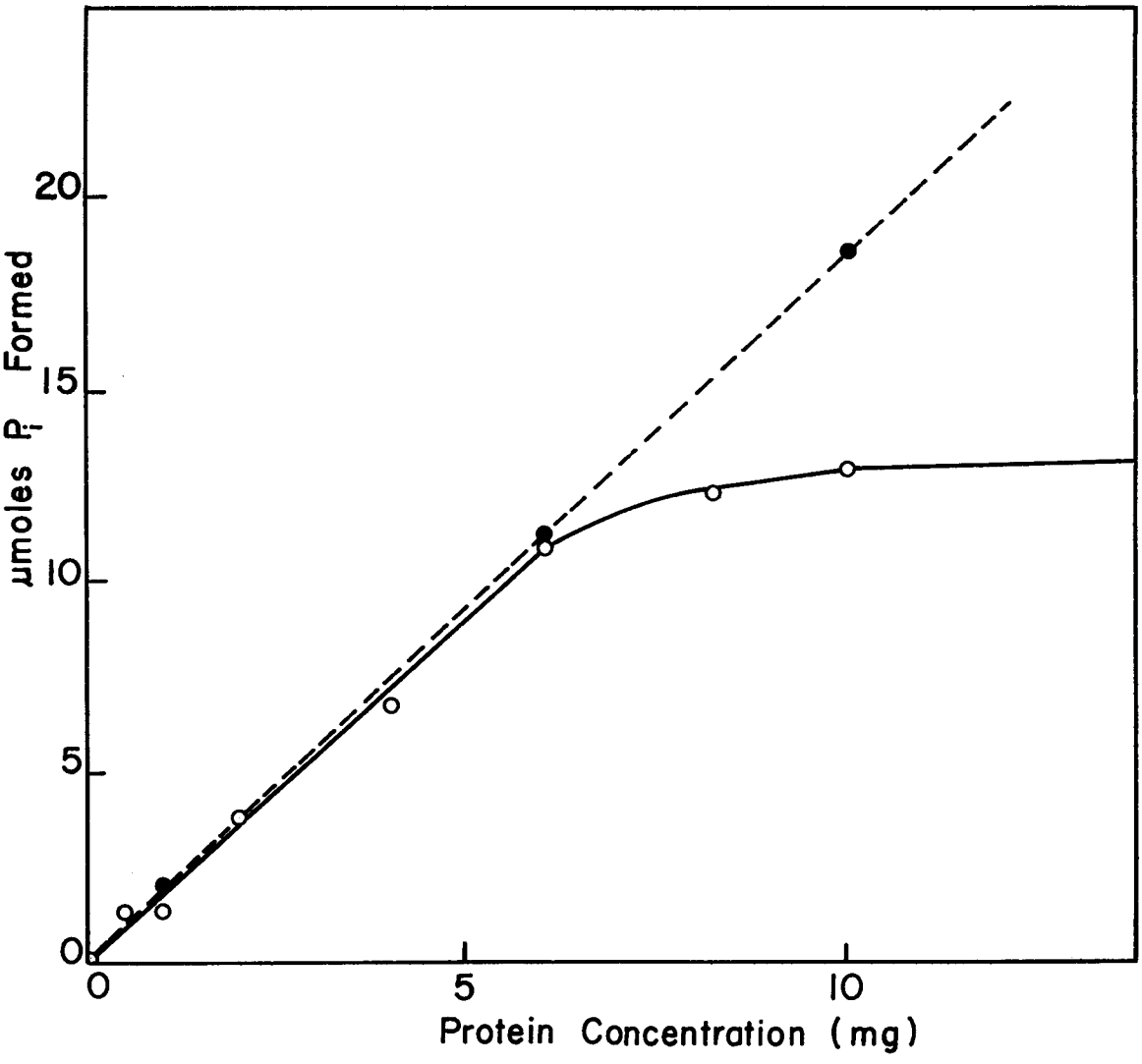
FIGURE 10

greater in the presence of, than in the absence of, the ATP regenerating system. This observation is similar to the findings of Pullman et al. (64, p. 3322-3327) with DNP stimulation of the purified ATPase from beef heart mitochondria.

Effect of Enzyme Concentration. Figure 11 shows that the increase in activity of the ATPase with increasing enzyme concentration is greatly enhanced in the presence of the ATP regenerating system, while in its absence, the activity rapidly reaches a limiting value. The results indicate that after an initial proportionality of enzyme concentration and Pi formation, the excess ADP accumulating in the medium competes for the active site on the enzyme and results in a suppression of ATPase activity. The linearity of formation of Pi with enzyme concentration can be maintained, however, if the ATP is removed as soon as it is formed, and this is effected through the enzymatic rephosphorylation of ADP in the presence of pyruvic kinase and phosphoenolpyruvate.

Effect of Substrate Concentration. Enzyme activity was found to increase linearily with increasing ATP concentration up to a concentration of 3 μmoles/ml. where the activity began to level off, reaching a maximum of about 8 μmoles/ml.

FIGURE 11



The Effect of Protein Concentration on ATPase Activity

Each flask contained 20 μmoles ATP, pH 7.2; 200 μmoles tris-acetate buffer, pH 7.2; 15 μmoles $MgCl_2$; indicated enzyme protein as a 1.0 ml. addition; and, where present 5 moles phosphoenolpyruvate and 32 μg pyruvic kinase in a final volume of 2.0 ml. Incubation time was for 30 minutes at 30° C. The results are the average of duplicate flasks. (0——0) Indicates the presence and (0- - -0) the absence of an ATP regenerating system.

ATP as shown in Figure 12. Concentrations greater than 10 μmoles/ml. were found to be inhibitory to the ATPase. There is a rather broad optimum substrate concentration between 6 and 8 μmoles ATP per mg. enzyme protein per ml. incubation medium at pH 7.2 and 30° C.

Effect of Temperature. The temperature for optimum ATPase activity was found to be in the range 20-30° C. with maximum activity at 25° C. as shown in Figure 13. The enzyme is rapidly inactivated at temperatures exceeding 30° C. and is completely inactive at 45° C. or higher. The presence of ATP at 1 x 10^{-3} M was found to afford some protection during heating to temperatures in excess of 40° C. but in general the enzyme lost 20% or more of its activity during only brief (2 minute) exposures to temperatures higher than 40° C. while in the presence of ATP. These results are shown in Table XI. Numerous combinations of various concentrations of ATP, Mg^{++}, sulfhydryl reagents and other known "protective" agents were employed unsuccessfully in attempts to stabilize the enzyme to heat denaturation.

Substrate Specificity. The substrate specificity of the purified ATPase was examined and as can be seen in Table XII

FIGURE 12

The Effect of Substrate Concentration on ATPase Activity

Each flask contained the indicated amount of ATP, pH 7.2; 200 μmoles tris-acetate buffer, pH 7.2; 15 μmoles $MgCl_2$; and 1.0 ml. enzyme solution containign 0.9-1.0 mg. protein/ml. in a final volume of 2.0 ml. Incubation time was 30 minutes at 30° C. The results are the average of duplicate flasks.

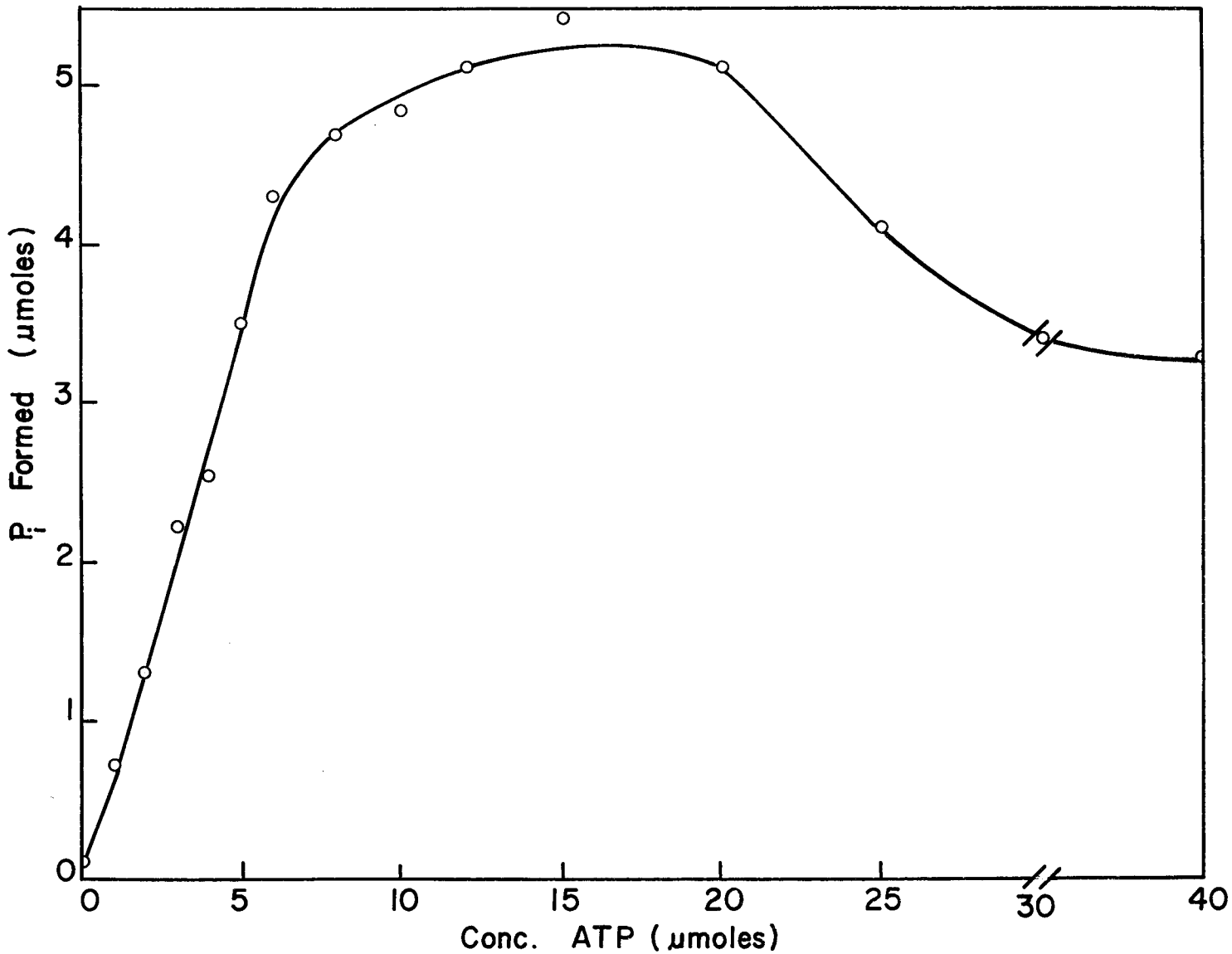
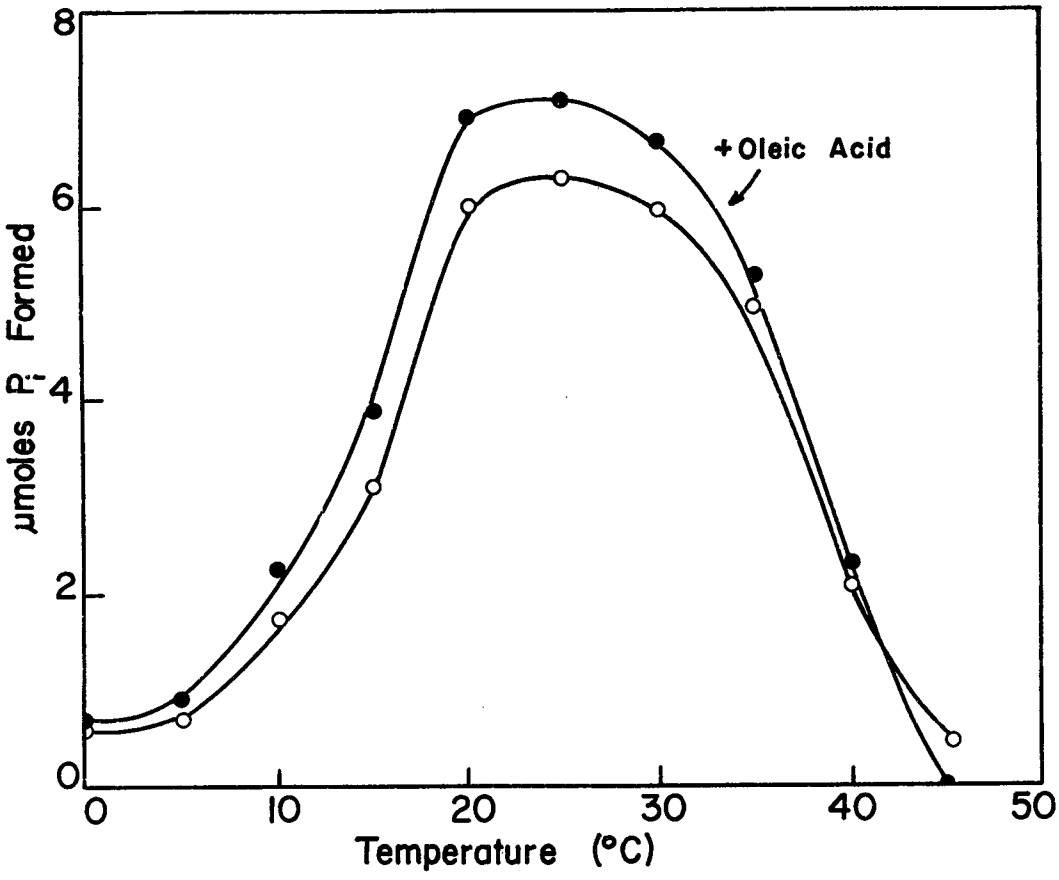

P_i Formed (μmoles)
0
1
2
3
4
5
0
5
10
15
20
25
30
40
Conc. ATP (μmoles)

FIGURE 13

The Effect of Temperature on ATPase Activity



The incubation medium is the same as that described in Figure 12 except that ATP was present at a concentration of 10 μmoles/ml., and, where indicated, oleic acid was present at 1 x 10^{-3} M. Incubation time was 30 minutes at the indicated temperature.

TABLE XI

The Effect of ATP in Preventing ATPase Denaturation by Heat

Heat Treatment	Presence of 1×10^{-3} M ATP	ATPase Activity moles Pi formed/mg. protein/ 30 min.
none	-	5.7
"	+	5.7
40°	-	4.7
40°	+	4.7
45°	-	4.0
45°	+	4.9
50°	-	1.2
50°	+	2.0
55°	-	0.30
55°	+	1.60

The incubation conditions are the same as those described in Table IX.

TABLE XII

Substrate Specificity of the ATPase

Substrate	moles Pi formed		
	No. Additions	+ 5 x 10^{-3} M Mg^{++}	+ 5 x 10^{-3} M Mg^{++} + 1 x 10^{-3} M oleic acid
ATP	0.87	3.28	4.44
ITP	3.04	5.18	5.82
GTP	1.48	2.38	2.30
UTP	1.80	2.22	2.20
CTP	2.30	1.80	1.80
Pyrophosphate	0.74	2.54	2.87

Each flask contained 6.0 μmoles nucleotides or pyrophosphate, pH 7.2; 100 μmoles tris-acetate buffer, pH 7.2; 5 μmoles $MgCl_2$; 0.5 mg. enzyme protein; and, where indicated, 1 x 10^{-3} M oleic acid in a final volume of 1.0 ml.

Incubation was for 10 minutes at 30° C. The results are averages of duplicate flasks.

all of the nucleoside triphosphates and inorganic pyrophosphate are split by the enzyme at significant rates. Such lack of substrate specificity among purified ATPase preparations has been demonstrated by numerous other workers. The 100-fold purified soluble ATPase from beef heart mitochondria (64, p. 3322-3329) likewise exhibited a lack of specificity for every nucleoside triphosphate tested except CTP, which was completely inactive. It was demonstrated that only the hydrolysis of ATP was stimulated by DNP. In the present study it was found that oleic acid stimulated the hydrolysis of ATP at the greatest rate (26%). Only ITP (11%) and pyrophosphate (12%) were affected, the rest being inactive towards hydrolytic stimulation by oleic acid. The enzyme preparation appears to be contaminated with several other phosphatase activities, including a Mg^{++}-stimulated inorganic pyrophosphatase. This activity does not appear to be markedly stimulated by oleic acid. It should be noted that the hydrolysis of ATP is more dependent upon the presence of added Mg^{++} than any other substrate tested.

Stoichiometry of Reaction. The 15-fold purified enzyme was shown to contain essentially no apyrase activity or organic

pyrophosphatase activity on the basis of chromatographic determination of the nucleotides present in the reaction mixture following incubation of crude and purified enzyme preparations with ATP. The purified ATPase was found to catalyze the hydrolysis of ATP to ADP and Pi, with no detectable AMP appearing. On the other hand, a less purified preparation (3-fold) contained an activity which catalyzed the formation of detectable quantities of AMP as well as Pi and ADP under the same conditions. That this activity in the less purified preparation may be an apyrase has been demonstrated by Mazelis (48, p. 153-158) who showed that cytoplasmic fractions of cabbage contain an appreciable amount of this enzyme. The amount of AMP appearing in the crude enzyme reaction mixture was estimated to lie between the limits of 1.5μ moles and 3.0μ moles AMP for each 20μ moles ATP initially present in the reaction mixture.

Thus, it can be concluded that with the 15-fold purified enzyme the reaction

$$(6)\quad ATP \longrightarrow ADP + Pi$$

must be the predominant pathway for the formation of inorganic phosphate since the reactions catalyzed by apyrase

$$(7)\quad ATP \longrightarrow AMP + 2Pi$$

or myokinase (adenylate kinase)

$$(8)\quad ATP + AMP \leftrightarrow 2ADP$$

could not be important because there was no significant level of AMP present in the reaction mixture as described above. On the other hand, the 3-fold purified enzyme must contain significant levels of enzyme (or enzymes) capable of forming AMP. Since it is clear from Table XII that a Mg^{++} stimulated inorganic pyrophosphatase is present even in the 15-fold purified enzyme, there exists the possibility that the ADP formed in Equations (6) and (8) would be hydrolyzed to inorganic phosphate as follows:

$$(9)\quad ADP \longrightarrow AMP + Pi$$

since inorganic pyrophosphatases need not be necessarily specific for inorganic pyrophosphate linkages. That Equation (9) does not occur to an appreciable extent in the 15-fold purified enzyme can be assumed on the basis of the lack of AMP present in reaction mixtures of this enzyme and ATP as described earlier.

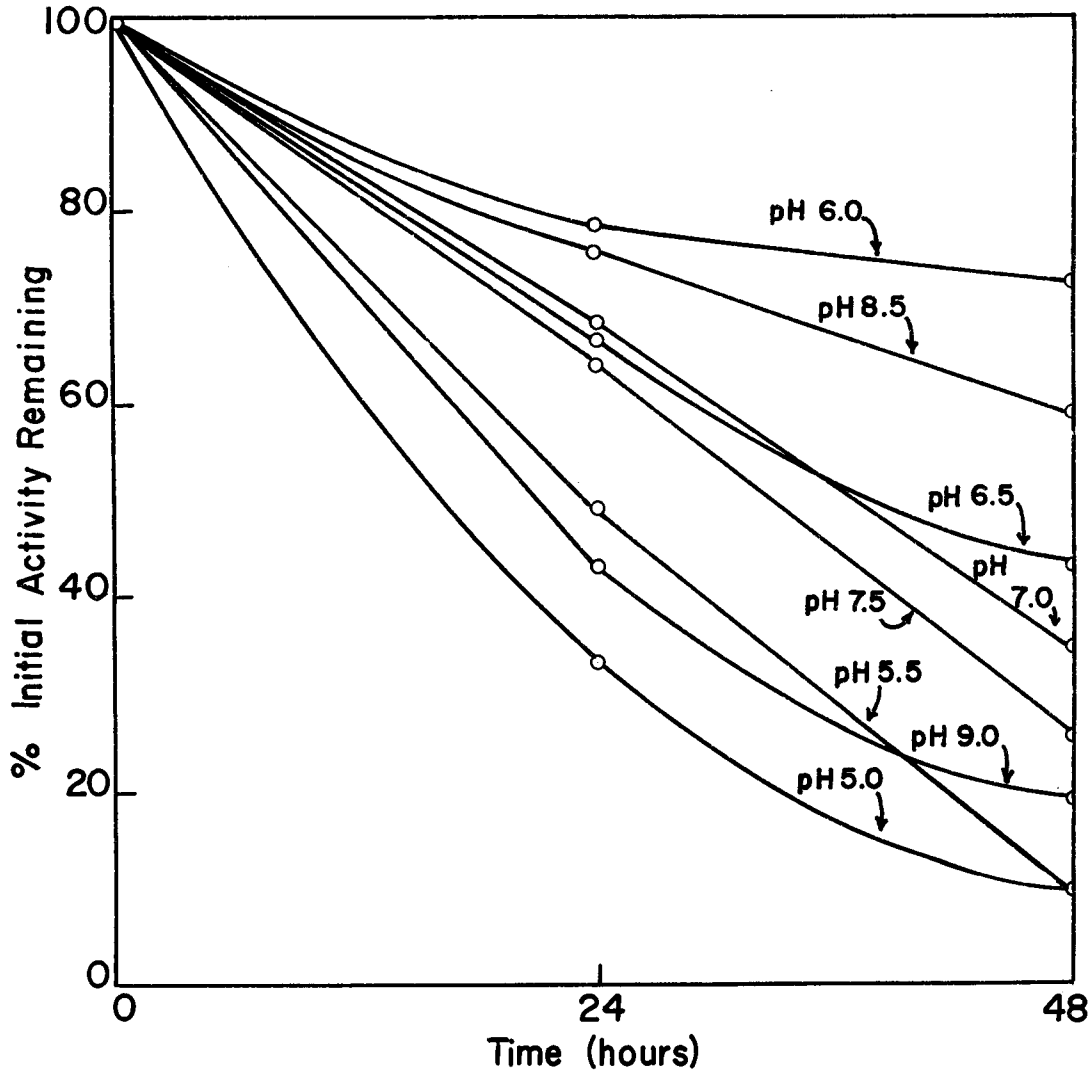
This reasoning may be used to explain the observed difficulty in achieving a significant increase in activity from the soluble supernatant step of both enzyme purification procedures described earlier. Since the 3-fold purified

enzyme contains enzymes capable of catalyzing Pi formation by virtue of Equation (7) as well as Equation (6), it follows that the observed enzyme activity would appear higher, on the basis of the amount of Pi formed, than warranted in terms of a specific cleavage of the terminal phosphate group of ATP as depicted in Equation (6). The 15-fold purified enzyme -- shown to be essentially free of the activity catalyzing Equation (7)-- may thus be considerably more purified in terms of a specific ATPase activity than is evident by relying upon the production of Pi as the criterion for enzyme activity. On this argument it is clear that more work is needed on the stoichiometry of the ATPase reaction in order to determine exact, rather than apparent purification factors.

Enzyme Stability. During the early studies on the ATPase it was noted that the enzyme was not stable in aqueous solution for periods exceeding 12 hours. The enzyme usually lost approximately 50% of its activity in 24 hours at 4° C. and at physiological pH. For this reason a detailed stability study was performed in order to determine the optimum storage conditions for the purified ATPase. Figure 14 suggests that the enzyme has maximum stability at pH 6.0 and rapidly loses activity as neutrality is approached, becoming

FIGURE 14

The Effect of pH on ATPase Stability



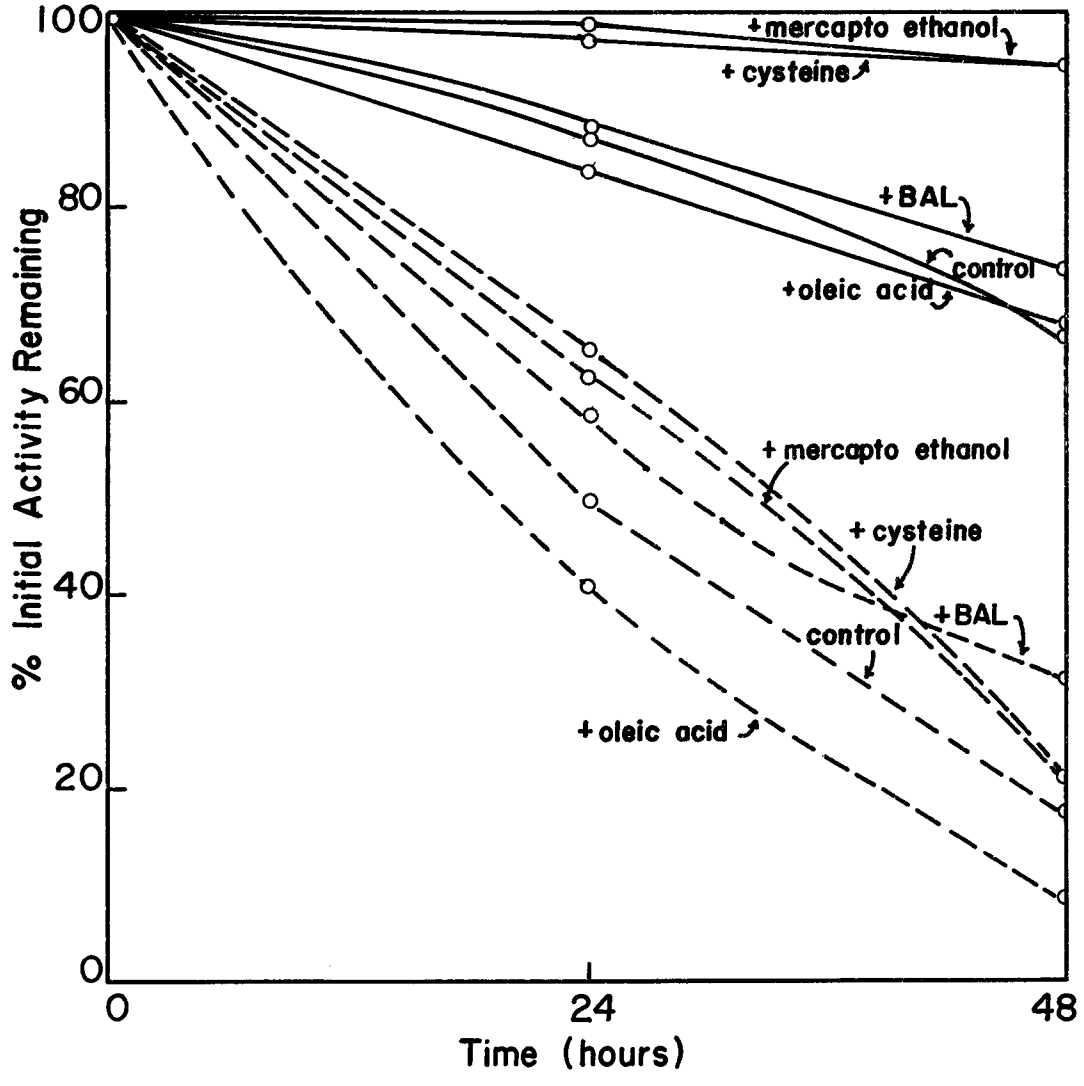
Enzyme solutions containing 0.9-1.0 mg. protein/ml. were stored for the indicated times at the designated pH values and then readjusted to pH7.2 and incubated 10 minutes as described in Figure 13 except that the temperature was 30° C.

increasingly stabilized up to pH 8.5. At pH values more acid than 6.0 or more alkaline than 8.5 the enzyme is inactivated. Thus the enzyme is most stable at pH values somewhat removed from neutrality. From Figure 14 it can be seen that at pH 7.5 the enzyme loses 50% of its activity after 24 hours and 75% of its activity after 48 hours, while only 22% and 28% of the activity was lost after 24 and 48 hours, respectively, at pH 6.0.

In order to explore further the problem of protecting the ATPase activity during prolonged storage, a number of substances known to protect enzymes from inactivation during isolation and storage were tested for their effects on preserving enzyme activity. Among the substances used were DMS (dimethyl sulfoxide), oleic acid, BAL, cysteine, mercaptoethanol, glycerol, glutathione, ascorbic acid, ATP, Mg^{++}, EDTA, and various combinations of the above. The results of this study are described in Figure 15. Mercaptoethanol and cysteine , each at a concentration of 5×10^{-3} M, appear to afford the best protection to the enzyme during storage at pH 6.0. As can be seen from Figure 15 the enzyme activity is rapidly lost at 24° C. in contrast to the cold labile ATPase described by Pullman et al. (64, p. 3322-3327).

FIGURE 15

The Effect of Various Substances on ATPase Stability



(0——0) indicates 4° C. storage and (0---0) 24° C. storage, both at pH 6.0. The incubation conditions are the same as those described in Figure 14.

The data shown in Figure 15 represent only the interrelationships among the substances found to impart appreciable stability to the enzyme during prolonged storage. All of the other substances tested were incapable of imparting any significant protection to the enzyme during storage.

The enzyme was also found to be completely inactivated by freezing, even in the presence of the previously mentioned protective agents employed either alone or in various combinations.

During the stability study it was found that the enzyme could be precipitated from aqueous solution by the slow addition of dry ammonium sulfate to 70% saturation at pH 6.0 in the presence of 5×10^{-3} M cysteine (or mercaptoethanol) and 1×10^{-3} M ATP. The enzyme could be kept for at least a week in this fashion without any appreciable loss in activity, whereas similar precipitation by ammonium sulfate in the absence of these protective agents and without adequate maintenance of the pH near 6.0 resulted in a considerable loss of activity. It is possible that the region near pH 6.0 may be a critical area for maximum stability of the enzyme and, this being the case, the failure of earlier attempts at storing the enzyme as an ammonium sulfate precipitate at

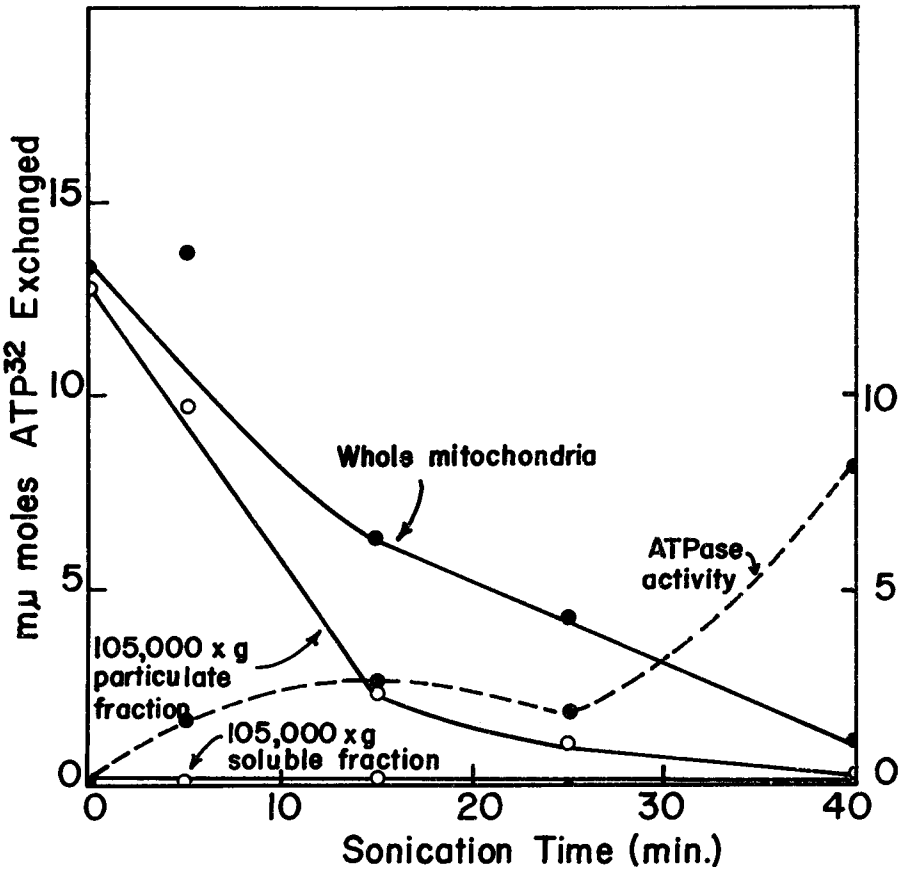
pH 5.5 (the pH of 70% ammonium sulfate solution) might be explained in terms of pH inactivation at this slightly greater acidity.

Upon redissolving the precipitated enzyme in a suitable buffer prior to incubation, it was necessary to remove a slight amount of residual sulfhydryl reagent and ammonium sulfate in order to regain maximum activity. The most effective means of doing this was by Sephadex G-25 chromatography as described in the section on "Methods and Materials". Dialysis at pH 6.0 against dilute buffer, although removing contaminants, was more time consuming and generally resulted in a loss of activity.

Lack of ATP-Pi^{32} Exchange Reaction. Chiga and Plaut (12, p. 3059-3066) have described an enzyme of mitochondrial origin that can catalyze an ATP-Pi^{32} exchange and which can be visualized as a reversal of Reactions (3) and (4) presented in the "Introduction". It can be seen from Figure 16 that sonic treatment rapidly destroys the enzyme catalyzing the ATP-P^{32} exchange in the mitochondrial particulate fraction and essentially no exchange activity is present in the soluble fraction. On the other hand, the soluble ATPase activity generally increases with increasing sonication

FIGURE 16

The Effect of Sonic Treatment Time on the ATP-Pi^{32} Exchange Reaction and ATPase Activity



Each flask contained 30 μmoles ATP, pH 7.4; 300 μmoles tris-maleate buffer, pH 7.4; 30 μmoles $MgCl_2$; 1.5×10^{-3} mg. bovine serum albumin; P^{32} equivalent to 9.6×10^5 counts/minute; 1.0 ml. mitochondrial enzyme preparation containing 14.9 mg. protein/ml. and glass redistilled water to a final volume of 3.0 ml. Incubation time was 30 minutes at 30° C.

time. In another experiment (with the 15-fold purified ATP-ase from acetone powder) it was similarly shown that there was no ATP-Pi^{32} exchange activity associated with the enzyme. These results are consistent with the findings of numerous other workers.

SUMMARY

A soluble, Mg^{++}-dependent ATPase from cabbage mitochondria has been partially purified from crude mitochondrial preparations obtained by differential centrifugation of tissue homogenates. The enzyme can be prepared by two different procedures: one employs sonic disintegration of mitochondria at pH 6.0 followed by ammonium sulfate precipitation of the 105,000 x g supernatant fraction; the other employs a DEAE-cellulose column chromatographic fractionation of soluble extracts obtained from an acetone powder prepared from intact cabbage mitochondria. Both procedures result in an ATPase activity approximately 15-fold purified and susceptible to stimulation by low concentrations of DNP (1×10^{-5} M to 5×10^{-5} M) and oleic acid (1×10^{-3} M). The purified enzyme was completely devoid of activity catalyzing the ATP-Pi^{32} exchange reaction.

Evidence is presented which suggests the possible involvement of an intermediate in oxidative phosphorylation. Eluant fractions from DEAE-cellulose chromatography of soluble acetone powder extracts were shown to contain, a non-dialyzable, acid-labile source of inorganic phosphate that occurred in the same fractions as the major ATPase

activity. The possible significance of this observation is discussed.

The enzyme activity was found to be associated with an intense, non-dialyzable yellow color independent of the presence of a flavin. When incubated with ATP and Mg^{++} at pH 7.2-7.4 the enzyme catalyzed a rapid cleavage of ATP to form inorganic phosphate and ADP. The stoichiometery of the reaction was not quantitatively determined; however, the enzyme was found to be quite non-specific in its activity towards other nucleoside-5'-triphosphates and inorganic pyrophosphate. Only the hydrolysis of CTP was not stimulated by the presence of 5×10^{-3} M Mg^{++}. Oleic acid at 1×10^{-3} M stimulated the hydrolysis of ATP and ITP but had no appreciable effect on UTP, GTP and CTP hydrolysis. The effect of DNP on the hydrolysis of these compounds was not studied.

Less purified ATPase preparations (3-fold purified) were shown to possess a significant apyrase or other enzymic activity capable of catalyzing the formation of AMP and Pi from ATP, but the 15-fold purified enzyme was shown to be essentially devoid of such activity. These findings may indicate that the enzymic purification factors cited are apparent,

and not absolute values in terms of the stoichiometery of the ATP hydrolysis reaction.

The enzyme was stimulated appreciably in the presence of an ATP regenerating system. The rate of ATP hydrolysis preceeded linearly with time and enzyme concentration whereas without the ATP regenerating system no such linearity could be demonstrated at higher concentrations or with longer incubation periods. This supports the finding that ADP inhibited the ATPase activity. 2,4-D at concentrations of 1×10^{-6} M to 1×10^{-5} M stimulated the enzyme, but higher concentrations were inhibitory. These results are different than those obtained from studies of 2,4-D on whole mitochondria.

The optimum substrate concentration for the enzyme was between 6μmoles and 8μmoles ATP/mg. enzyme protein at pH 7.2 and 30° C. Concentrations of ATP in excess of 10μmoles/mg. enzyme protein were inhibitory to the enzyme.

The purified enzyme exhibited an unusual behavior with pH in that it possessed two and possibly three distinct pH optima, a major one at pH 7.0-7.2 and two minor ones at pH 6.4-6.6 and pH 7.5-7.7. The enzymic activity was irreversibly destroyed at pH values less than 5.0 or greater

than 9.5. The activity could be protected down to pH 4.7 in the presence of 1 x 10^{-3} M ATP and 5 x 10^{-3} M cysteine.

Mg^{++} was found to be an obligatory cofactor for the enzyme, eliciting maximum activity at a concentration of 5 x 10^{-3} M. The ATP/Mg^{++} ratio resulting in the greatest enzyme activity was 1:1, a ratio different from that found for other ATPases in mammalian systems.

The purified ATPase was maximally active at a temperature between 25° and 30° C. and was irreversibly denatured at temperatures in excess of 45° C. The presence of 1 x 10^{-3} M ATP or other protective agents were of some value in stabilizing the enzyme at higher temperatures (up to 55° C.), but a significant amount of activity was usually lost. The enzyme lost 100% of its activity upon freezing and thawing, even in the presence of ATP, sulfhydryl reagents, dimethyl sulfoxide, glycerol, or other substances known to protect enzymes during adverse treatment.

The enzyme was found to be maximally stabilized in precipitated form at 4° C. in 70% ammonium sulfate solution, pH 6.0, containing 5 x 10^{-3} M cysteine (or mercaptoethanol) and 1 x 10^{-3} M ATP. The activity could also be maintained in aqueous solution for at least 48 hours at pH 6.0 and in

the presence of ATP and sulfhydryl reagent as described above with only slight loss of activity, whereas in the absence of added reagents and at pH 7.4, 50% of the activity was lost in 24 hours at 4° C.

Some of the results obtained from the study of the solubilized ATPase are at variance with the characteristics of the enzyme limited to the confines of the intact mitochondrion. It is possible that this aberrant behavior may be explained in terms of a physical change conferred upon the solubilized enzyme that is absent in the enzyme bound to the highly organized mitochondrial structure.

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